Tesis Doctoral

ESPECIALIZACIÓN EN MICORRIZAS: FACTORES ECOLÓGICOS E IMPLICANCIAS MACROEVOLUTIVAS

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Índice de Materias

RESUMEN

El mutualismo ha tenido un papel central en la ecología y evolución de la biodiversidad. Esta interacción se caracteriza por presentar una gran variación en el nivel de especialización, que, debido a sus potenciales efectos a escala evolutiva y ecológica, es de gran interés para biólogos evolutivos y ecólogos. La especialización podría ser favorable si resulta en una mayor eficiencia en el intercambio de recursos y/o servicios, pero al mismo tiempo podría implicar una menor capacidad de interactuar con otras especies y así persistir frente a cambios ambientales, llevando a los linajes especialistas a un "callejón sin salida" evolutivo. Pese a sus posibles desventajas, existen numerosos casos de especialización en la naturaleza, lo que sugiere que, bajo ciertas condiciones ecológicas, la especialización podría ser ventajosa o neutral. Para entender cuáles son las implicancias evolutivas de la especialización y a una escala ecológica, cuáles son aquellos factores que promueven la especialización, en esta tesis se estudió la especialización en las micorrizas, una asociación entre plantas y hongos del suelo que está presente en la mayoría de las plantas terrestres y presenta una gran variación en el nivel de especialización. De esta manera, en esta tesis se abordaron las siguientes preguntas (1) ¿Qué efecto tiene la especialización micorrícica sobre las tasas de diversificación de las plantas? (2) ¿Qué factores ambientales promueven la especialización micorrícica? Para responder la primera pregunta, el enfoque se centró en la historia evolutiva de las micorrizas, estudiando los cuatro tipos de micorriza a lo largo de la evolución de las plantas. Con esto, se evaluó la relación entre la diversidad de tipos de micorrizas y las tasas de diversificación de las familias de plantas con semillas, demostrándose que una mayor diversidad de tipos de micorrizas se relaciona con mayores tasas de diversificación. Estos resultados apoyan la hipótesis de la especialización como una estrategia

desfavorable a escala evolutiva y corroboran la importancia de las asociaciones micorrícicas en la evolución de las plantas. Para la segunda pregunta, en cambio, nos centramos en un tipo particular de micorrizas, las micorrizas de orquídeas, donde se estudió el papel de los factores ambientales, específicamente los nutrientes del suelo, en la especialización micorrícica. Utilizando una especie de orquídea endémica de Chile, *Bipinnula fimbriata*, se evaluó (1) el efecto de la disponibilidad de nutrientes en la comunidad de hongos asociados a plantas adultas de *B. fimbriata* en una población natural y (2) el efecto de los nutrientes en el inicio de la asociación simbiótica, que ocurre en el encuentro del hongo micorrícico con la semilla de la orquídea. Ambos estudios mostraron que los nutrientes tienen un papel clave en las asociaciones micorrícicas de *B. fimbriata*, tanto en plantas adultas como en la etapa de la germinación, mostrando además que en plantas adultas los nutrientes pueden afectar indirectamente a toda la comunidad de hongos asociados (incluidos hongos micorrícicos y no micorrícicos). Los resultados sugieren además que el efecto de los nutrientes en la especialización micorrícica depende de la escala espacial, en el caso de poblaciones naturales, y de la identidad del hongo involucrado, en el caso de la germinación simbiótica. Esta tesis aporta con nuevos conocimientos sobre la interacción entre orquídeas y hongos, abre numerosas preguntas para futuras investigaciones y contribuye a la comprensión de la importancia de la asociación con hongos en la estructuración de las comunidades y la evolución de las plantas.

ABSTRACT

Mutualisms have a central role in the ecology and evolution of biodiversity. Mutualisms are characterized by a high variation in specialization, which, due to its potential effects at evolutionary and ecological scale, is of great interest to ecologist and evolutionary biologist. Specialization might be advantageous if it results in a higher efficiency in resource/services exchanges. In turn, it could result in a lower capacity to shift partner under environmental fluctuations, leading specialist lineages to "evolutionary dead-ends". Despite of this potential disadvantages, specialization is common in nature, which suggests that under certain environmental conditions, specialization could be advantageous or neutral. In order to understand the evolutionary implications of specialization and -at the ecological level- the factors that promote specialization, in this thesis I studied the specialization in mycorrhizas, the association between plants and soil fungi that is present in most of plants species. In this way, address the following questions (1) How mycorrhizal specialization affects the plants diversification rates? and (2) Which environmental factors promote mycorrhizal specialization? To answer the first questions, focused was placed on the evolutionary history of mycorrhizas, studying the four main types of mycorrhizas along the evolution of plants. Thus, the relationship between diversity of mycorrhizal types and diversification rates of seed plant families was assessed, which showed that a higher diversity on mycorrhizal types was associated with higher diversification rates. These results support the hypothesis that specialization has negative implications at the evolutionary scale and confirm the importance of mycorrhizal associations in the evolution of plants. For the second question, we focused in a particular type of mycorrhizas, the orchid mycorrhiza, where we studied the role of environmental factors, especially soil nutrients, in mycorrhizal specialization. Using an

endemic orchid species, *Bipinnula fimbriata*, we evaluated (1) the effect of nutrient availability on the fungal root community associated with adult plants of *B. fimbriata* in a natural population and (2) the effect of nutrients in the initial contact between the mycorrhizal fungi and the orchid seed. Both studies showed that nutrients play a key role in mycorrhizal associations of *B. fimbriata*, both in adult plants and in the germination, in adult plants it was also observed that nutrients can affect indirectly the whole fungal community (including mycorrhizal and non-mycorrhizal fungi). The results also suggest that the effect of soil nutrients in mycorrhizal specialization depends on the spatial scale, in the case of natural populations, and on the identity of the fungal species involved. This thesis contributes with new knowledge about orchid mycorrhizal associations, opens many novel questions for future research and contributes to the understanding of the importance of plants-fungal associations in the structuration of communities and the evolution of plants.

INTRODUCCIÓN GENERAL

El mutualismo, la interacción en la que individuos de diferentes especies se benefician mutuamente, ha tenido un papel central en la ecología y evolución de la Tierra. Grandes hitos evolutivos en la historia de la vida están relacionados con el mutualismo, como el origen de la célula eucarionte (Sagan, 1967), la colonización de la tierra por parte de las plantas (Pirozynski & Malloch, 1975) y la radiación de las angiospermas (Pellmyr, 1992). La influencia del mutualismo trasciende los niveles de organización biológica, desde la célula hasta poblaciones, comunidades y ecosistemas (Bronstein *et al* 2015). A nivel comunitario, por ejemplo, el mutualismo tiene un rol esencial en la estructuración de comunidades terrestres y acuáticas (Silliman *et al*., 2011, He *et al*., 2013) y a nivel ecosistémico, provee funciones ecosistémicas como la dispersión de frutos, polinización y ciclos de carbono, de nitrógeno y fósforo (Bronstein *et al*., 2015; Johnson *et al*., 2015).

Los mutualismos se caracterizan por presentar una gran variación en el nivel de especialización, donde las especies participantes forman un gradiente que va desde especies que interactúan solamente con una especie (especialistas) hasta especies que forman mutualismos con múltiples especies (generalistas) (Ollerton, 2006). Esta variación en el nivel de especialización ha interesado a biólogos evolutivos y ecólogos, debido a sus potenciales efectos a escala evolutiva y ecológica. La especialización podría ser ventajosa si resulta en una mayor eficiencia en el intercambio de recursos y/o servicios, a través de la pérdida de rasgos que son sólo necesarios para interactuar con un rango más amplio de especies (Futuyma & Moreno, 1988). Sin embargo, esta pérdida de rasgos podría ser irreversible y a una escala temporal mayor podría resultar en una menor capacidad de interactuar con otras especies y así persistir frente a cambios ambientales (Haldane, 1951). Además, la especialización podría

llevar a una menor probabilidad de encontrar una especie alternativa con la que interactuar ante fluctuaciones ambientales (Poisot *et al*., 2011). De esta forma, la especialización podría significar un "callejón sin salida" evolutivo, es decir, aquellos linajes que se especializan podrían tener mayores tasas de extinción -debido a una mayor vulnerabilidad frente a cambios ambientales- y menores tasas de especiación -debido a una menor diversidad de formas desde la cual generar nuevas especies-, y lo que resultaría en tasas de diversificación menores que los linajes generalistas, y en una capacidad muy limitada de revertir hacia interacciones generalistas (Day *et al*., 2016). Por otro lado, pese a sus posibles desventajas, existen numerosos casos de especialización en la naturaleza, lo que sugiere que, bajo ciertas condiciones, la especialización podría ser ventajosa o neutral (Day *et al*., 2016; Vamosi *et al.*, 2014). En este contexto, la biología de mutualismos busca entender cuáles son las implicancias evolutivas de la especialización y a una escala ecológica, cuáles son aquellos factores que promueven la especialización.

Para abordar ambas preguntas, en esta tesis se estudió la especialización en las micorrizas, uno de los mutualismos más comunes y ampliamente distribuidos del planeta (Smith & Read, 2008). Las micorrizas son una asociación entre hongos del suelo y las raíces de las plantas (Brundrett, 2002), donde las plantas entregan carbono a los hongos y los hongos a su vez le entregan nutrientes a las plantas (Marschner & Dell, 1994). Las micorrizas están presentes en el 86% de las especies de plantas terrestres (van der Heijden *et al*., 2015) y de acuerdo a su estructura y función pueden ser clasificados en cuatro tipos principales: Micorrizas Arbusculares (AM), Ectomicorrizas (EM), Micorrizas de Orquídeas (OM) y Micorrizas Ericoidales (ER) (Brundrett, 2002). Las asociaciones micorrícicas son en su mayoría generalistas, con plantas interactuando con un amplio rango de hongos micorrícicos

(Molina *et al*., 1992; Smith & Read, 2008). Sin embargo, existen plantas que interactúan con un rango restringido de especies de hongos y esta variación en el nivel de especialización ocurre tanto entre como dentro de los cuatro tipos de micorrizas (van der Heijden *et al*., 2015). La variación en el nivel de especialización, y su amplia distribución geográfica y taxonómica, que abarca una gran diversidad de gradientes ambientales y de linajes de plantas, hacen de las micorrizas un modelo ideal para estudiar la especialización en el mutualismo, sus implicancias evolutivas y los factores ecológicos que la promueven. De esta manera, en esta tesis se abordarán las siguientes preguntas:

(1) ¿Qué efecto tiene la especialización micorrícica sobre las tasas de diversificación de las plantas?

(2) ¿Qué factores ambientales promueven la especialización micorrícica?

Para responder la primera pregunta, el enfoque será puesto en la historia evolutiva de las micorrizas, estudiando los cuatro tipos de micorrizas a lo largo de la evolución de las plantas, con el fin de evaluar si aquellos linajes generalistas tienen tasas de diversificación mayores que los linajes especialistas (Capítulo I). Para la segunda pregunta, en cambio, se utilizará un tipo particular de micorrizas, las micorrizas de orquídeas, y utilizando una especie de orquídea endémica de Chile, *Bipinnula fimbriata*, se evaluará el papel de los factores ambientales en la especialización micorrícica (Capítulos II y III).

I. Implicancias macroevolutivas de la especialización en micorrizas

La reconstrucción ancestral de caracteres y el registro fósil muestran que el ancestro de las plantas con semillas probablemente tenía micorrizas arbusculares (AM) (Redecker *et al.*, 2002; Maherali *et al.*, 2016). Este es el tipo de micorriza más frecuente, está presente en el

74% de las plantas y se caracteriza por asociarse con hongos del phylum Glomeromycota (van der Heijden *et al.,* 2015). A partir del estado ancestral AM, algunos linajes de plantas han recorrido los siguientes caminos evolutivos: asociarse con un nuevo grupo de hongos (EM, OM y ER) o perder la asociación (NM) (Werner *et al.,* 2018). Las transiciones evolutivas a un nuevo tipo de micorrizas pueden haber permitido a las plantas acceder a recursos ecológicos inexplorados, facilitándolos para colonizar ambientes que antes no estaban disponibles y posiblemente incrementar sus tasas de diversificación. Sin embargo, hay algunos linajes que además de adquirir un nuevo tipo de micorriza, retienen el estado ancestral (AM) (Brundrett, 2008) incrementando la variabilidad de tipos de micorrizas, lo que podría promover también la diversificación de esos linajes. Ambas hipótesis no han sido evaluadas, a pesar de que la simbiosis con hongos micorrícicos ha sido señalada como un factor clave en la diversificación de las plantas (Brundrett & Tedersoo, 2018; Feijen *et al.,* 2018).

En el primer capítulo de la tesis, por lo tanto, se evaluaron las siguientes hipótesis (1) Los linajes que establecen un nuevo tipo de micorriza (EM, OR, ER o NM) tienen mayores tasas de diversificación que aquellos linajes que mantienen el estado ancestral (AM) y (2) Aquellos linajes que poseen una mayor variabilidad en el tipo de micorriza poseen mayores tasas de diversificación. Para poner a prueba estas hipótesis, se evaluó la relación entre los tipos de micorrizas y las tasas de diversificación de las familias de plantas con semillas. Para esto, se compiló una base de datos de 6450 especies de plantas con semilla y su tipo de micorriza. A partir de esta información se asignó un tipo de micorriza a cada familia, se calculó la heterogeneidad de tipos de micorrizas y se estimaron las tasas de diversificación utilizando el método de Magallón & Sanderson (2001).

II. Factores que promueven la especialización en micorrizas: *Bipinnula fimbriata* (Orchidaceae) como modelo de estudio

Las comunidades de hongos micorrícicos están fuertemente influenciadas por factores bióticos y abióticos, como el clima (Tedersoo *et al*., 2012), las características del suelo (Treseder, 2004; Bunch *et al*., 2013; Huggins *et al*., 2014) y la identidad de la planta (Roy *et al*., 2013). En particular, la disponibilidad de nutrientes en el suelo influye en el nivel de especialización y composición de hongos micorrícicos en AM y EM (Lilleskov *et al*., 2002; Parrent *et al*., 2006; Polme *et al*., 2013; Roy *et al*., 2013) y puede afectar negativamente la colonización micorrícica, que estaría relacionada con la riqueza de hongos micorrícicos (Blechem & Alexander, 2012; Balzergue *et al*., 2013).

El rol que juegan los nutrientes del suelo en las micorrizas de orquídeas es bastante más desconocido. Orchidaceae, la familia más diversa de las Angiospermas (Chase *et al*., 2015), forma un tipo exclusivo de micorrizas, llamado micorrizas de orquídeas. Las orquídeas producen semillas extremadamente pequeñas que carecen de reservas energéticas (Arditti and Ghani, 2000; Barthlott *et al*., 2014), y dependen completamente de los hongos micorrícicos para la germinación. En este proceso- conocido como "germinación simbiótica"- el hongo coloniza la semilla y juntos forman un cuerpo indiferenciado no fotosintético llamado protocormo, que depende completamente del hongo para su nutrición (Rasmussen, 2002; Kuga *et al.,* 2014). Luego se desarrollan las primeras hojas, las plántulas pasan a ser autotróficas (en el caso de las orquídeas fotosintéticas) y ocurre el intercambio de nutrientes entre el hongo micorrícico y la orquídea (Cameron *et al.,* 2006; Perotto *et al*., 2014). Los hongos que forman micorrizas con las orquídeas pertenecen a tres familias (Tulasnellaceae,

Sebacinaceae and Ceratobasidiaceae) de los Basidiomycetes (Dearnaley *et al*., 2012), sin embargo, la diversidad y composición de los hongos micorrícicos de orquídeas (HMO) varía considerablemente entre especies de orquídeas y entre distintos hábitats dentro de una misma especie (Pandey *et al*., 2013; Jacquemyn *et al*., 2015).

Estudios recientes han mostrado que las comunidades de HMO pueden variar en función de la variación temporal o del hábitat (Oja *et al*., 2015; Cevallos *et al*., 2018) y la limitada evidencia sugiere que los nutrientes del suelo podrían tener un papel importante en esta variación (Bunch *et al*., 2013; Mujica *et al*., 2016). En un estudio anterior, se investigaron las asociaciones micorrícicas de *Bipinnula fimbriata*, una especie de orquídea endémica de Chile que forma densas poblaciones en zonas costeras de Chile central, y se observó una relación negativa entre la disponibilidad de nutrientes y la diversidad HMO asociados a las raíces de esta orquídea (Mujica *et al*., 2016). Sin embargo, dado que este estudio fue correlacional y la diversidad de hongos fue evaluada aislando hongos en cultivo *in vitro*, son necesarios estudios experimentales que utilicen técnicas de secuenciación masiva (independientes de cultivo *in vitro*) para comprobar el rol de los nutrientes en las asociaciones micorrícicas de *B. fimbriata*. Por esto, en el segundo capítulo se llevó a cabo un experimento de fertilización en una población extensa de *B. fimbriata*, con el fin de evaluar el efecto de la adición de nutrientes sobre el nivel de especialización micorrícica en esta especie de orquídea.

Por otro lado, no están claros los mecanismos que podrían explicar la relación entre los nutrientes del suelo y la diversidad de hongos micorrícicos asociados a *B. fimbriata*. Una alternativa es que esta relación se deba a un efecto de los nutrientes sobre la germinación simbiótica. Es posible que la capacidad germinadora de los hongos varíe en función de los

nutrientes disponibles (McCormick *et al*., 2018) y que esto se vea reflejado en la diversidad de hongos asociados a las plantas adultas. Estudios previos han demostrado que la adición de nutrientes afecta negativamente la germinación simbiótica en orquídeas (Beyrle *et al*., 1991, 1995); sin embargo, se desconoce si este efecto varía entre diferentes especies de hongos micorrícicos, ni los mecanismos subyacentes. Debido a que la composición y diversidad de HMO asociados a las orquídeas varía en función de las condiciones climáticas y edáficas (McCormick *et al*., 2006; Bunch *et al*., 2013, Mujica *et al*., 2016; Reiter *et al*., 2018); y a que los HMO tienen diferentes requerimientos nutricionales (Hadley & Ong, 1978; Nurfadilah *et al*., 2013; Fochi *et al*., 2017); se espera que (1) el efecto de la adición de nutrientes sobre la germinación varíe dependiendo de la identidad del hongo, y (2) que esta variación se relacione con las preferencias nutricionales de los hongos. Para poner a prueba ambas hipótesis, en el tercer capítulo se evaluó el efecto de la identidad del hongo micorrícico, la adición de nutrientes y su interacción sobre la germinación simbiótica de *Bipinnula fimbriata*. Para esto se utilizaron hongos micorrícicos aislados de plantas adultas de *B. fimbriata* y semillas esterilizadas de esta misma especie, y se evaluó la germinación simbiótica y la tasa de crecimiento de los hongos bajo diferentes concentraciones de nutrientes.

III. Objetivos de Tesis

Objetivo general

Analizar los factores ecológicos y las implicancias evolutivas de la especialización en las asociaciones micorrícicas

Objetivos específicos

(1) Evaluar la relación entre la diversidad de tipos de micorrizas y las tasas de diversificación de las familias de plantas con semilla
(Capítulo I)

(2) Examinar el papel de los nutrientes del suelo en el nivel de especialización micorrícica de la orquídea *Bipinnula fimbriata*
(Capítulo II)

(3) Examinar el papel de los nutrientes y de la identidad del hongo micorrícico en la germinación simbiótica de *Bipinnula fimbriata*
(Capítulo III)

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Capítulo I: Seed plant families with diverse mycorrhizal states have higher diversification rates

Seed plant families with diverse mycorrhizal states have higher diversification rates

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SUMMARY

One crucial innovation in plant evolution was the association with soil fungi during land colonization. Today, this symbiotic interaction is present in most of plants species and can be classified in four types: Arbuscular (AM), Ecto (EM), Orchid (OM) and Ericoid Mycorrhiza (ER). Since the AM ancestral state, some plants lineages have switched partner (EM, OM and ER) or lost the association (no-association: NM). Evolutionary transitions to a novel mycorrhizal state (MS) might allow plant lineages to access new resources, enhancing diversification rates. However, some clades are not restricted to one MS, and this variability might promote diversification. In this study we address the relationship between MS and plant diversification rates of seed plant families. For this, we compiled a database for ~6400 seed plant species and their mycorrhizal partners. We assigned a single MS to each plant family, then calculated the heterogeneity of MS and estimated their diversification rates using the method-of-moments. Families with mixed MS had the highest diversification rates and there was a positive relationship between heterogeneity of MS and diversification rates. These results support the hypothesis that MS plasticity promotes diversification and highlight the importance of the association with soil fungi for the diversification of plants.

Keywords: *diversification rates, mycorrhizal states, seed plants, key innovation, mycorrhizal diversity*

INTRODUCTION

Understanding the basis of the exceptional plant diversity has been a matter of interest for ecologist and evolutionary biologist since Darwin. Great focus has been placed on estimating plants diversification rates and identifying the factors that could influence them (Eriksson & Bremer, 1992; Moore & Donoghe, 2007; O´Meara *et al*., 2016; Vamosi *et al.,* 2018). The acquisition of novel traits (sometimes referred to as "key innovations"), such as pollination by animals (Eriksson & Bremer, 1992) or physiological seed dormancy (Willis *et al.,* 2014), have been proposed to promote diversification of plant lineages. This "key innovation" perspective suggests that the acquisition of a novel trait might allow a given lineage to exploit the environment in a significantly different way, potentially resulting in an explosive radiation.

One crucial innovation in plants evolution was the association with soil fungi during land colonization (Pirozynski & Malloch, 1975; Selosse & Le Tacon, 1998). Before plant colonization, land was hostile, with extreme drought and temperatures, and barren rocky substrate; hence, the association with terrestrial fungi allowed the algae ancestors of plants to successfully colonize the land (Selosse *et al.,* 2015). This initial symbiotic association was the prelude of modern mycorrhizas (Feijen *et al.,* 2018), the association between fungi and root plants in which plants transfer carbon to fungi and receive nutrients in turn (Smith & Read, 2008). Today, this symbiosis is present in 86% of land plants species (van der Heijden *et al.,* 2015), and based on their structure and function can be classified in four major types: arbuscular mycorrhiza (AM), ectomycorrhiza (EM), orchid mycorrhizal (OM) and ericoid mycorrhizal (ER) (Brundrett, 2002).

Ancestral state reconstruction and the fossil record show that the ancestor of seed plants probably had AM associations (Redecker *et al.,* 2002; Maherali *et al.,* 2016). This is the most frequent mycorrhizal type in plants (74% of extant plant species) and is characterized by an association with Glomeromycete fungi (van der Heijden *et al.,* 2015). Between 100 and 200 million years ago, some lineages switched fungal partners to several lineages of Basidiomycetes, forming what is described as the EM associations (Brundrett, 2002). The acquisition of EM resulted in new root functional capabilities as freezing tolerance (Lehto *et al*., 2008), which seem related to the dominance of EM angiosperms and gymnosperm in cool forests (Brundrett, 2002). Similarly, Orchidaceae and species within the Ericaceae family recruited new fungal lineages and formed OM and ER associations respectively. Orchids associate with fungal families Ceratobasidiaceae, Tulasnellaceae and Sebacinaceae, which in addition to nutrient exchange, promote seed germination which cannot germinate without mycorrhizal support (Rasmussen, 2002). Ericoid mycorrhizal associations (ER), on the other hand, involve mainly fungi from Sebacinales and Helotiales and are mostly frequent under acidic and infertile heathland conditions (Perotto *et al.,* 2002; van der Heijden *et al*., 2015). Finally, some lineages have lost their mycorrhizal associations and became non-mycorrhizal (NM). This transition has frequently occurred through an intermediate state of facultative arbuscular mycorrhiza (AM) plants (Maherali *et al.,* 2016). Some of NM lineages evolved alternative resource-acquisition strategies (Werner *et al.,* 2018) like cluster-roots in Proteaceae (Neumann & Martinoia, 2002) or parasitism in Loranthaceae (Wilson & Calvin, 2006).

Therefore, since the AM ancestral state some plant lineages have followed different mycorrhizal evolutionary pathways: switching partner (EM, OM and ER) or losing the association (Werner *et al.,* 2018). Evolutionary transitions to a novel mycorrhizal state might

allow plant lineages to access unexplored ecological resources, facilitating them to colonize environments that were not available before, and possibly enhancing their diversification rates. However, there are lineages in which some species acquire a new mycorrhizal state and at the same time, other species retain the ancestral state (AM) (Brundrett, 2008) increasing the variability of mycorrhizal states, which might in fact promote diversification of these lineages. Both hypotheses have not been evaluated in plants, however the few studies available from the fungal perspective suggest that shifts in mycorrhizal associations might affect diversification of involved partners (Sánchez-García & Matheny, 2017; Sato *et al.*, 2017).

Even though mycorrhizal symbiosis has been pointed out as a key factor in the evolution and diversification of land plants (Brundrett & Tedersoo, 2018a; Feijen *et al.*, 2018) this has not been evaluated before. In this study we address the following questions: (1) Do the lineages that established specific mycorrhizal associations differ in their diversification rates? This investigates the idea of a key innovation mechanism of diversification; (2) Is there a relationship between mycorrhizal variability and diversification rates among different plant lineages? This would investigate the idea that evolutionary lability might increase diversification dynamics. To answer these questions, we explored the relationship between the mycorrhizal state and the diversification rates of several seed plants families.

MATERIALS AND METHODS

Mycorrhizal state database

We used the species-level dataset of mycorrhizal status from Maherali *et al.*, (2016), which compiles previous lists and surveys of plant species and their mycorrhizal associations. Then, to increase sample size, we reviewed publications that report mycorrhizal states for

single species, species list or local vegetation. Our literature compilation resulted in a database of 6440 species and their mycorrhizal state (Supporting Information, Notes S1). We used Maherali *et al.,* (2016) classification, and assigned species into one of these categories: arbuscular mycorrhizal (AM), ectomycorrhiza (EM) Orchid mycorrhizal (OM), Ericoid mycorrhizal (ER) and Non-mycorrhizal (NM). Species that were characterized as AMNM by Maherali *et al.,* (2016) - i.e. species observed as AM in some environments and NM in others- were here considered as AM as they correspond to facultative AM species. Also, as Maherali *et al.,* (2016), species that formed both AM and EM, were placed in the EM category to account species that were potentially capable of forming EM symbiosis. The species names were reviewed using the Taxonomic Name Resolution Service (tnrs.iplantcollaborative.org). Recently, Brundrett & Tedersoo (2018b) pointed out potential mistakes in mycorrhizal type identification on large databases, and how these misdiagnoses might lead to wrong conclusions. For the case of Maherali *et al.,* (2016) database, the authors estimated an error in 1.6% genera and 1.0% species, which in the grand scheme of things seems small and unlikely to produce biased results. Moreover, their approach used to determine these errors (taxonomic approach; Brundrett, 2017) is controversial (Bueno *et al.,* 2018). Nevertheless, to assess the effect of possible undetected errors in the mycorrhizal dataset, we introduced errors to the mycorrhizal state of 20% of plant species (one order of magnitude higher than the error estimated from Brundrett & Tedersoo 2018b) and obtained similar results to those derived from original data (Supporting Information, Table S1).

Family mycorrhizal state and diversity

We obtained information for species belonging to 259 seed plant families, although the species sampling among families was highly variable. To reduce the chance of wrongly

assigning a family mycorrhizal state, we considered those families for which we had either 5% or higher of species sampled or at least 8 species sampled. This is justified because although many families are species-rich, they also seem to be quite consistent with respect to mycorrhizal association (Brundrett, 2008). This reduced our dataset to 175 families. Each family was assigned a unique mycorrhizal state (AM, EM, NM, ER or OM) when more than 60% of species sampled belonged to this mycorrhizal state. If no single state were present in more than 60% of species, the family was assigned a "mixed" state, to indicate no dominance of any mycorrhizal association. Other thresholds for the assignment of family mycorrhizal state were tested and the pattern was similar (50%, 80% and 100%, Table S2 and Fig. S1). To investigate the effect of mycorrhizal diversity in the diversification dynamics we estimated the "Mycorrhizal diversity index", which is calculated by estimating the heterogeneity of the mycorrhizal states in each family using the shannon diversity index.

Diversification rates

Diversification rates for each seed plant family were estimated using the method-of-moments from Magallón & Sanderson (2001). Because the relative contribution of extinction is unknown we used distinct scenarios to characterize the relative extinction rates (ε), one with no extinction, $\varepsilon = 0.0$, one with medium extinction, $\varepsilon = 0.5$, and another with high extinction, $\varepsilon = 0.9$. Following Stadler et al (2014) we used crown group ages instead of stem ages, given that those result in more reliable estimates. We also excluded monotypic families from the analyses, for which crown group age is unavailable. Crown group ages of the families were obtained from the dated molecular phylogeny of seed plants of Zanne *et al.*, (2014) by obtaining the most recent common ancestor of all species within each family. Given that crown group ages might be affected by species under-sampling, only the families that had

more than 60% of the genera sampled in the phylogeny were included in the analyses. Further analyses were performed with different thresholds for % of genera and similar trends were found (50, 80 and 100%; Table S3 and S4) The number of species of each family was obtained from The Plant List (theplantlist.org). We are aware of more sophisticated and direct methods (e.g. BAMM; Rabosky, 2014) to investigate the association between trait states and diversification dynamics, but the plant phylogeny is massively under-sampled at the species level, and we clearly do not have mycorrhizal information for most species. Therefore, we decided to use simpler and less data hungry methods, and to discuss our results in the light of the methods limitations.

Phylogenetic signal

The seed plant phylogeny (Zanne *et al.*, 2014) was pruned to obtain a family level phylogeny, with one species per family as tips. From this pruned phylogeny we calculated the phylogenetic signal of mycorrhizal traits and diversification rates. For the continuous variables - mycorrhizal diversity index and diversification rates - we calculated phylogenetic signal using Pagel's Lambda (Pagel, 1999) using the function phylosig in the package phytools in R (Revell, 2012). For the categorical variable, mycorrhizal state, we estimated the phylogenetic signal using the D parameter (Fritz & Purvis, 2010) with the function phylo.d in caper package in R (Orme *et al.*, 2013).

Statistical analysis

As some (but not all) of the mycorrhizal traits and diversification rates showed significant phylogenetic signal (Table S5), we evaluated the effect of mycorrhizal associations on diversification rates by both considering and not the phylogenetic structure in the residuals. We tested for potential differences in diversification rates between plant families with

different mycorrhizal types using both ANOVA and a phylogenetic ANOVA using the function phylanova from phytools in R. Each mycorrhizal state was used as group and their diversification rates as response variable. Because the mycorrhizal states OR and ER only had one family each, those were removed from this analysis. To test for the relationship between mycorrhizal heterogeneity and diversification rates we performed a linear model with raw data, and a PGLS regression in the R package caper (Orme *et al.*, 2013) with diversification rates as response variable and mycorrhizal heterogeneity as explanatory variable. For PGLS models we used the lambda value obtained from the previous phylogenetic signal analysis. To further test if any specific mycorrhizal state promote diversification, we follow the approach taken by Moen & Wiens (2017) and evaluated the correlation between the proportion of each mycorrhizal state in the family and their diversification rate (Fig S4). To further explore the potential confounding effect and the association between mycorrhizal association and diversification dynamics, we performed PGLS regressions to assess the relationship between mycorrhizal diversity index, age and species richness.

Finally, if shifts in mycorrhizal states (MS) occurred only once within each family (e.g. species within sub-clades within each family all have the same MS), the family level analyses might not properly capture the effects of mycorrhizal shifts on diversification rates. To explore whether mycorrhizal shifts in mixed families might have occurred multiple times, we calculated the proportion of MS within genera of mixed families and mapped them in the phylogeny of each mixed family to check if MS form monophyletic sub-clades (Fig. S5).

RESULTS

We obtained information about mycorrhizal state of 6441 species that belong to 259 families of seed plants. According to our sampling criteria, we kept 175 families and then, after excluding monotypic families and families with less than 60% of the genera sampled in the phylogeny (for which crown group age is unavailable or underestimated), we kept 106 families. From these 106 families, 80 were AM (for example, Amaryllidaceae, Cupressaceae and Euphorbiaceae), 11 were EM (as Fagaceae and Pinaceae), 15 were NM (such as Ceratophyllaceae and Juncaginaceae) and 7 were mixed (Fig. 1). Mixed families contain species that retained the ancestral state (AM) and species that present a different mycorrhizal state (EM or NM). There were two types of mixed families: two mixed families had AM, EM and NM species, (Myrtaceae and Cyperaceae) while the other five had AM and NM species (Amaranthaceae, Anisophylleaceae, Bromeliaceae, Juncaceae and Montiaceae) (Table S8).

The phylogenetic signal strength differs among mycorrhizal types. While AM and EM are mostly spread randomly across the plant phylogeny, NM and MIX are phylogenetically clustered to some extent (Table S5). Likewise, the phylogenetic signal of diversification rates was significantly different from a random structure in $r^{\varepsilon=0.0}$ and $r^{\varepsilon=0.5}$ but not in $r^{\varepsilon=0.9}$ (Table S5). There was a significant difference in diversification rates between the mycorrhizal states, irrespective of the extinction scenario ($r^{\varepsilon=0.0}$ $F=8.9$, $P=2.5x10^{-5}$; $r^{\varepsilon=0.9}$ $F=9.7$, $P=1x10^{-5}$; Fig. 2), which was observed in the ANOVA and in the phylogenetic ANOVA (Table S6). The *a posteriori* analysis of the ANOVA showed that diversification of MIX families were significantly higher than that of AM, EM and NM families. The same tendency is observed when correcting for the phylogenetic structure (Table S7). The phylogenetic ANOVA also

showed there was no significant difference in diversification rates between the two types of mixed families ($r^{\varepsilon=0.0}$ F=0.07, P=0.75; $r^{\varepsilon=0.9}$ F=0.6, P=0.36).

The higher values of mycorrhizal diversity index were found in Nyctaginaceae (1.088), Polygonaceae (0.926), Phyllanthaceae and Myrtaceae (0.88 and 0.75 respectively), while the lowest was zero and it was observed in 86 families that have all species in the same mycorrhizal state, like in Pinaceae (EM, n=140), Araucariaceae (AM, n=9) and Bignonaceae (AM, n=20). There was a positive correlation between mycorrhizal diversity index and diversification rates, observed with the linear models and with the PGLS (Figure 3a and 3b). The r^2 are surprisingly high, and together with the p-values of the models, are shown in Table 1. The significant relationship is observed under the three different scenarios of extinction (Table 1, only $r^{\varepsilon=0.0}$ and $r^{\varepsilon=0.9}$ are shown in Fig. 3). Mycorrhizal diversity index had no correlation with age and a significant but very low correlation with species richness (Fig. 3e, 3f). There was no correlation between the proportion of any specific mycorrhizal type in the family and their diversification rate (Fig. S4).

DISCUSSION

The association with mycorrhizal fungi has been indicated as a key acquisition in the evolution of plants, nevertheless its effect on plants diversification has not been evaluated before. Here we presented the first attempt to assess the relationship between mycorrhizal associations and diversification rates of plants. Due to the under-sampling of seed plants phylogeny and mycorrhizal state database, we used a simple and conservative approach that allows us to tackle this question.

Our results showed that families with mixed mycorrhizal type show higher diversification rates than AM, EM and NM families (Fig. 2). Mixed strategy included two subtypes of mixed: families with AM and NM species, and families with AM, EM and NM species; both had higher diversification rates and there was no significant difference on rates between them. This shows that regardless of the mycorrhizal states that composed the mixed families, they have the highest diversification rates, suggesting that it is the diversity of mycorrhizal states that promotes diversification rather than a specific mycorrhizal state. This is further supported by the fact that there was no correlation between the proportion of any specific mycorrhizal type in the family and their diversification rate.

In addition, there was a positive and significant correlation between mycorrhizal diversity index and diversification rates, which does not depend on our categorical criteria of mycorrhizal state assignment to families. These associations with diversification rates, are both observed when correcting or not for the phylogenetic structure, suggesting that the relationship is not due to phylogenetic relatedness between families. Also, the patterns are observed under different scenarios of extinction, and even with ε=0.9, where extinction could have an important role, the relationship is conserved. Given that diversification rates are determined by age and richness of the family, the effect of those variables could have driven the relationship between mycorrhizal heterogeneity and diversification rates. We observed no significant correlation between mycorrhizal heterogeneity and age; and we see a similar pattern with species richness, although the correlation is significant, the r^2 is quite low (Fig. 2e, 2f). This supports that mycorrhizal heterogeneity is mainly associated with diversification rates, not with age nor richness per se.

Both results, the ANOVA for family mycorrhizal type and correlation between mycorrhizal heterogeneity and diversification, suggest that independent of which mycorrhizal state is involved, a higher heterogeneity of mycorrhizal states in a family might promote diversification rates. We interpret mycorrhizal heterogeneity as a result from a higher evolutionary lability of the mycorrhizal states within these families, which has been suggested to promote diversification in other biotic interactions (Hardy & Otto, 2014). Each mycorrhizal state provides advantages to plants in certain environments but not in others (Brundrett *et al.*, 2002), thus families that are composed by species with different mycorrhizal states might have been able to switch states in evolutionary time, making them able to evolve a higher diversity of niches which would result in a higher diversification rate. Under this scenario, mycorrhizal diverse families would have had more chances to take advantage of a new ecological opportunity, than families with most species within a single mycorrhizal state. It is interesting to note that mycorrhizal diverse families have not only higher diversification when compared to low diverse families with the ancestral state, but also higher rates than families that have switched from the ancestral state to one novel mycorrhizal state (NM and EM families).

The mycorrhizal diversity index might not capture well the effects of mycorrhizal shifts on diversification rates if shifts occurred only once within each Family. However, we observed that mycorrhizal shifts in mix families occurred multiple times, because the MS do not form monophyletic sub-clades and shifts occur even below the genus level. This clearly suggest that diversification rates are not the result of a single mycorrhizal shift, but a result of high lability of the mycorrhizal types within the mixed families (Fig. S5). These results together suggest that rather than a key innovation scenario, it is the evolutionary variability of mycorrhizal state what promotes diversification rates of plant seed families. Our results also

highlight the evolutionary role of specialization at different organization levels: even if species are mycorrhizal specialized within a mixed family, the possibility to switch to different mycorrhizal states might increase the diversification of the family.

Because biodiversity dynamics could be rather complex, with clades either expanding, at equilibrium and even declining in diversity, simple metrics like the average rate of diversification might not be able to separate them (Quental & Marshall, 2010). The use of an average rate as a descriptor of a clade diversification dynamics assumes (or at least equates to) a scenario of expanding diversity (Quental & Marshall, 2010), and it might be especially problematic if lineages have a carrying capacity because the average rate might be diluted as time goes by (Rabosky, 2009). Moreover, with an average rate is not possible to distinguish between speciation and extinction rates or to test directly the effect of one trait on diversification dynamics. Ideally one would use more complex tests, but that would require a lot more phylogenetic data than what is currently available. Additionally, the ecological data is scarce, and the identification of root associations might be complicated by inconsistent applications of definitions (Brundrett, 2008). Thus, our study points out the need for more accurate ecological knowledge on plants species and their mycorrhizal state.

Acknowledging the limitations of our study, the results suggest that a higher diversity of mycorrhizal strategies promotes diversification of lineages, possibly related with new ecological opportunities that each mycorrhizal state provides to plants. Our results finally suggest that the associations between soil fungi and plants has been key for plant diversification, not only due to the foundational association that allows plants colonize land (Pirozynski & Malloch, 1975) but also for further diversification of seed plant lineages.

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AUTHOR CONTRIBUTIONS

MIM, TQ and MFP designed the research; MIM collected the data, GB, MIM and TQ analyzed the data, GB made the figures; and all the authors wrote the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Notes S1. Attached file with the mycorrhizal state database.

Fig. S1. Relationship between mycorrhizal type and diversification rate estimated with ε= 0 and with ε= 0.9, using different filters for MIX state assignment.

Fig. S2. Relationship between mycorrhizal type and diversification rate estimated with ε= 0 and with ε= 0.9, using different filters for crown group age estimation.

Fig. S3. Relationship between mycorrhizal diversity index and diversification rate estimated with ε = 0 and with ε= 0.9, using different filters for crown group age estimation.

Fig. S4. Relationship between the proportion any specific mycorrhizal type in the family and their diversification rate (estimated with ε= 0 and with ε= 0.9).

Fig S5. Genus-level phylogenies of mixed families showing proportion of mycorrhizal states within each genus at the tips.

Table S1. Relationship between family mycorrhizal type (ANOVA), mycorrhizal diversity index (linear model) and diversification rates, using a modified dataset that contains 20% of randomly selected species with wrong mycorrhizal state assignation.

Table S2. Summary of ANOVAs between mycorrhizal type and diversification rate using different thresholds for mycorrhizal type assignation.

Table S3. Summary of ANOVAs between mycorrhizal type and diversification rate using different filters for crown group age estimations.

Table S4. Summary of correlations between mycorrhizal diversity index and Diversification rates using different filters for crown group age estimations.

Table S5. Phylogenetic signal of mycorrhizal traits and diversification rates

Table S6. Results of ANOVA and phylogenetic ANOVA between mycorrhizal type and diversification rate.

Table S7. *A posteriori* analysis of ANOVA and phylogenetic ANOVA

Table S8. Seed plant families included in the analyses, % of species with each mycorrhizal state (AM, arbuscular mycorrhiza; EM, ectomycorrhiza and NM, non-mycorrhizal), family mycorrhizal state, diversification rates and mycorrhizal diversity index.

FIGURE LEGENDS

Figure 1. Family-level, time-calibrated phylogeny for the 106 seed plant families included in the analyses. For each family, the proportion of species within each mycorrhizal type is represented in the yellow-to-red boxes, AM: Arbuscular mycorrhiza, EM: Ectomycorrhiza and NM: non-mycorrhizal. The mycorrhizal diversity index (MDI) is represented in the green boxes and the diversification rate (r) is shown in the purple boxes. To illustrate the timescale of the phylogeny, the width of concentric white and gray circles represents 100 million years.

Figure 2. Relationship between mycorrhizal type and diversification rates. a) diversification rate estimated with ε (relative extinction fraction) = 0 and b) diversification rate estimated with ε= 0.9. AM: Arbuscular mycorrhiza, EM: Ectomycorrhiza, NM: non-mycorrhizal and MIX (families with no dominance of any specific mycorrhizal association).

Figure 3. Scatterplots showing the relationship between mycorrhizal diversity index and diversification rates, species richness and age family. The red and blue lines indicate the results of a linear model and a phylogenetic generalized least squares (PGLS) fit, respectively. b) and d) show the correlation between observed values of diversification rate and estimated values obtained from the PGLS (red line represents the perfect fit).

Table 1. Summary of PGLS and linear model between mycorrhizal diversity index (predictor variable) and diversification rate (response variable), for each of the estimated diversification rates analyzed separately. ε is the relative extinction fraction used for the estimation of diversification rate, and lambda is the estimated phylogenetic signal of the PGLS.

Fitting PGLS for Mycorrhizal diversity index			
Diversification rate	r^2	P-value	lambda
$r^{\varepsilon=0.0}$	0.19	1,19e-03	0.367
$r^{\varepsilon=0.5}$	0.20	4,96e-04	0.355
$r^{\varepsilon=0.9}$	0.25	2,68e-05	0.262
Fitting standard linear model for Mycorrhizal diversity index			
Diversification rate	r^2	P-value	
$r^{\varepsilon=0.0}$	0.19	1,11e-03	
$r^{\varepsilon=0.5}$	0.20	4,97e-04	
$r^{\varepsilon=0.9}$	0.24	2,95e-05	

Figure 1.

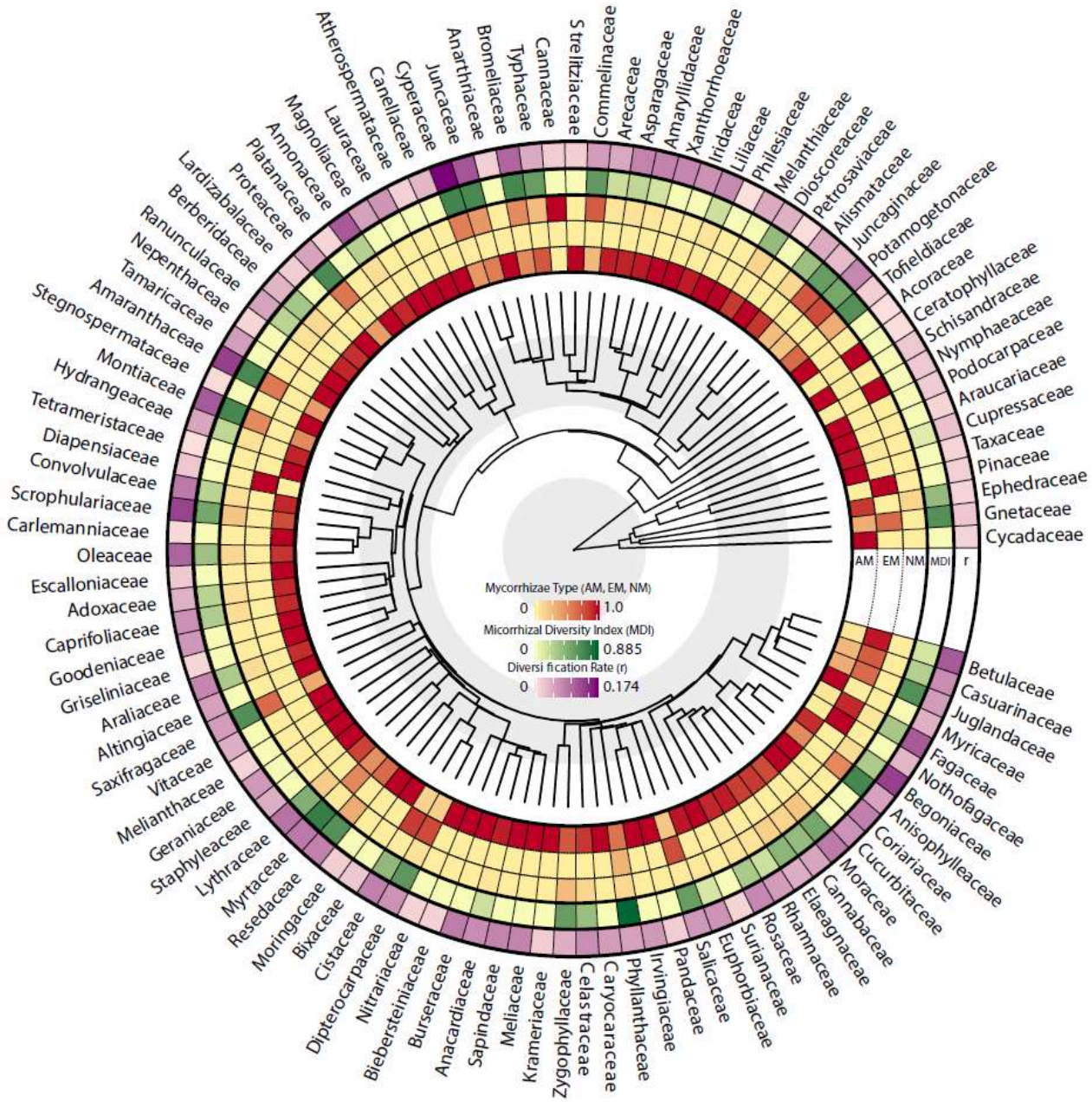

Mycorrhizae Type (AM, EM, NM)
0
1.0
Micorrhizal Diversity Index (MDI)
0
0.885
Diversi fication Rate (r)
0
0.174
AM
EM
NM
MDI
r
Cycadaceae
Gnetaceae
Ephedraceae
Pinaceae
Taxaceae
Cupressaceae
Araucariaceae
Podocarpaceae
Nymphaeaceae
Schisandraceae
Ceratophyllaceae
Acoraceae
Tofieldiaceae
Potamogetonaceae
Juncaginaceae
Alismataceae
Petrosaviaceae
Dioscoreaceae
Melanthiaceae
Philesiaceae
Liliaceae
Iridaceae
Xanthorrhoeaceae
Amaryllidaceae
Asparagaceae
Arecaceae
Commelinaceae
Strelitziaceae
Cannaceae
Typhaceae
Bromeliaceae
Anarthriaceae
Juncaceae
Cyperaceae
Canellaceae
Aetherospermataceae
Lauraceae
Magnoliaceae
Annonaceae
Platanaceae
Proteaceae
Lardizabalaceae
Berberidaceae
Ranunculaceae
Nepenthaceae
Tamaricaceae
Amaranthaceae
Stegnospermataceae
Montiaceae
Hydrangeaceae
Tetrameristaceae
Diapensiaceae
Convolvulaceae
Scrophulariaceae
Carlemanniaceae
Oleaceae
Escalloniaceae
Adoxaceae
Caprifoliaceae
Goodeniaceae
Griseliniaceae
Araliaceae
Altingiaceae
Saxifragaceae
Vitaceae
Melianthaceae
Geraniaceae
Staphyleaceae
Lythraceae
Myrtaceae
Resedaceae
Moringaceae
Bixaceae
Cistaceae
Dipterocarpaceae
Nitrariaceae
Biebersteiniaceae
Burseraceae
Anacardiaceae
Sapindaceae
Meliaceae
Krameriaceae
Zygophyllaceae
Celastraceae
Caryocaraceae
Phyllanthaceae
Irvingiaceae
Pandaceae
Salicaceae
Euphorbiaceae
Surianaceae
Rosaceae
Rhamnaceae
Elaeagnaceae
Cannabaceae
Moraceae
Cucurbitaceae
Coriariaceae
Anisophylleaceae
Begoniaceae
Nothofagaceae
Fagaceae
Myricaceae
Juglandaceae
Casuarinaceae
Betulaceae

Figure 2.

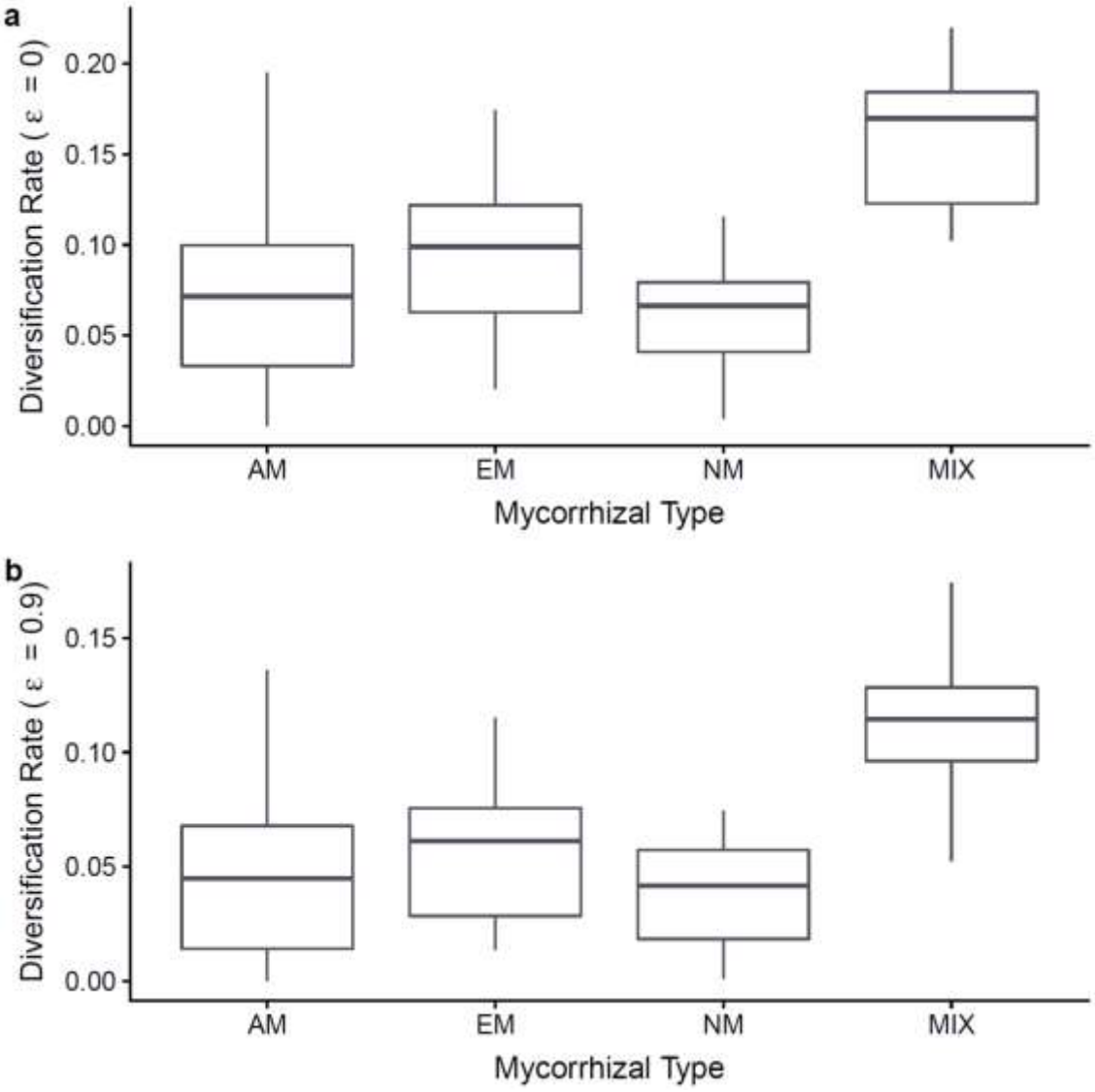
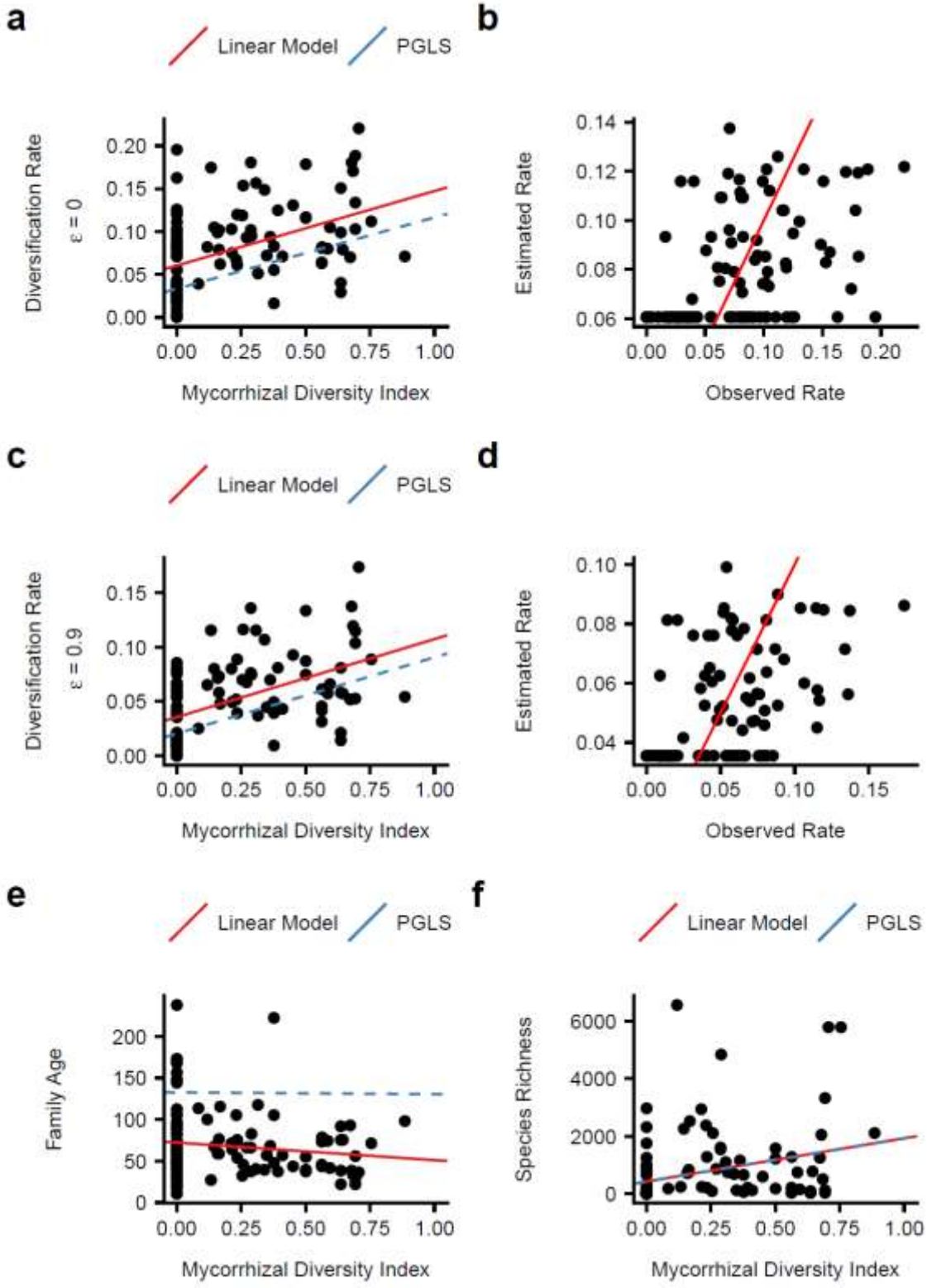

a
Diversification Rate (ε = 0)
0.20
0.15
0.10
0.05
0.00
AM
EM
NM
MIX
Mycorrhizal Type
b
Diversification Rate (ε = 0.9)
0.15
0.10
0.05
0.00
AM
EM
NM
MIX
Mycorrhizal Type

Figure 3.


a
Linear Model
PGLS
Diversification Rate
ε = 0
0.20
0.15
0.10
0.05
0.00
0.00 0.25 0.50 0.75 1.00
Mycorrhizal Diversity Index
b
Estimated Rate
0.14
0.12
0.10
0.08
0.06
0.00 0.05 0.10 0.15 0.20
Observed Rate
c
Linear Model
PGLS
Diversification Rate
ε = 0.9
0.15
0.10
0.05
0.00
0.00 0.25 0.50 0.75 1.00
Mycorrhizal Diversity Index
d
Estimated Rate
0.10
0.08
0.06
0.04
0.00 0.05 0.10 0.15
Observed Rate
e
Linear Model
PGLS
Family Age
200
150
100
50
0
0.00 0.25 0.50 0.75 1.00
Mycorrhizal Diversity Index
f
Linear Model
PGLS
Species Richness
6000
4000
2000
0
0.00 0.25 0.50 0.75 1.00
Mycorrhizal Diversity Index

Supporting Information

I. Introduced errors to the mycorrhizal state in dataset

Recently, Brundrett & Tedersoo (2018b) pointed out potential mistakes in mycorrhizal type identification on large databases, and how these misdiagnoses might lead to wrong conclusions. For the case of Maherali *et al*., (2016) database, the authors estimated an error in 1.6% genera and 1.0% species, which in the grand scheme of things seems small and unlikely to produce biased results. Moreover, their approach used to determine these errors (taxonomic approach; Brundrett, 2017) is controversial (Bueno *et al*., 2018). Nevertheless, to assess the effect of possible undetected errors in the mycorrhizal dataset, we introduced errors to the mycorrhizal state of 20% of plant species (one order of magnitude higher than the error estimated from Brundrett & Tedersoo, 2018b).

To do this, we randomly selected 20% of the plant species from the mycorrhizal state database and replace their mycorrhizal states with a different one. After this, we did the same analyses that we performed with the original data. The results obtained with the error-introduced database were similar than those derived from original data.

Table S1. Relationship between family mycorrhizal type (ANOVA), mycorrhizal diversity index (linear model) and diversification rates, using a modified dataset that contains 20% of randomly selected species with altered mycorrhizal state assignation.

Response variable	ANOVA Family mycorrhizal type		Correlation Mycorrhizal diversity index	
	F	p-value	Adjusted R^2	p-value
Mycorrhizal type vs Diversification rate (e=0.0)	3.0	0.03	0.1	4 E-04
Mycorrhizal type vs Diversification rate (e=0.9)	3.8	0.01	0.22	2 E-07

II. Thresholds for mycorrhizal type assignation to families

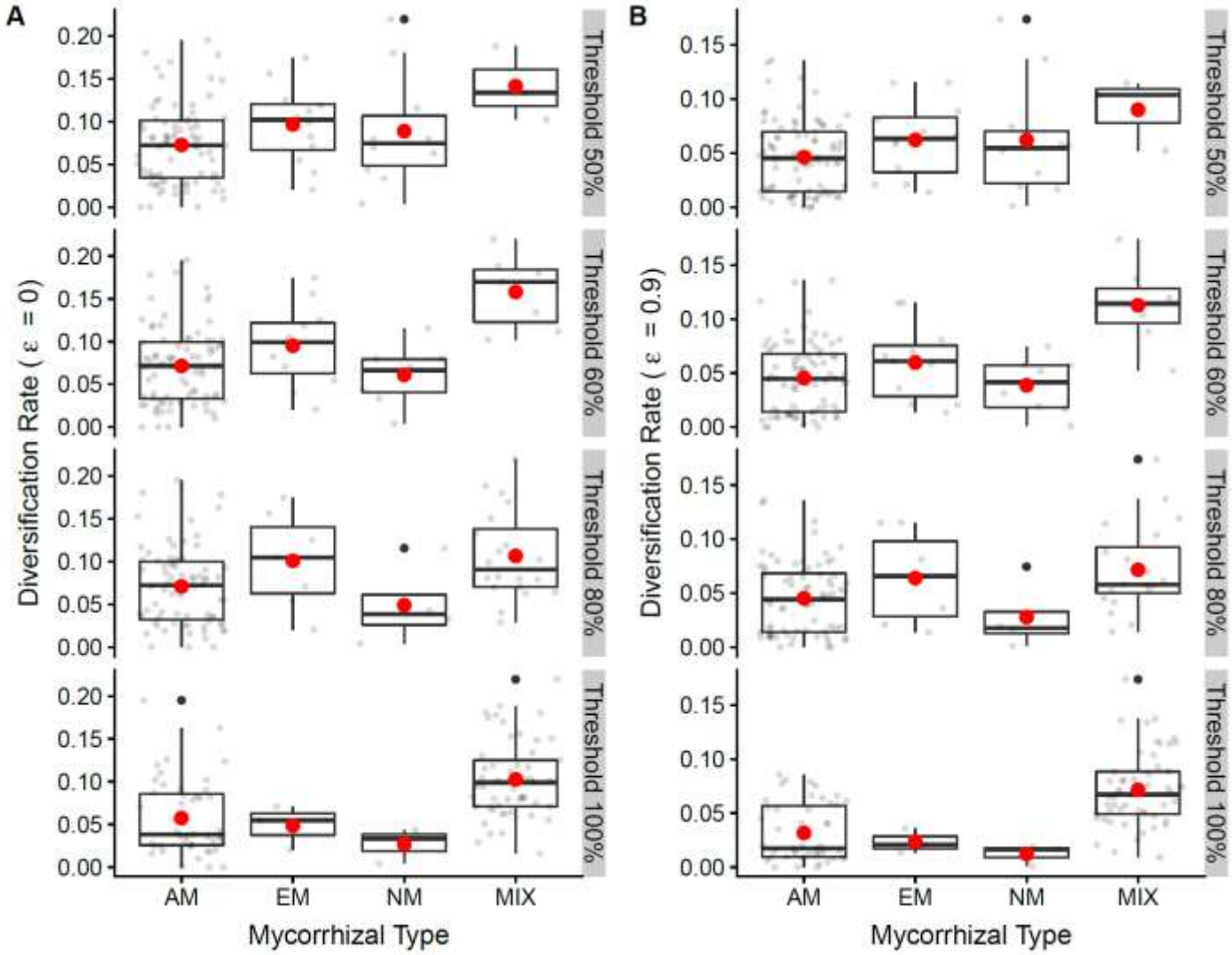
To each family was assigned a unique mycorrhizal state (AM, EM, NM, ER or OM) when more than 60% of species sampled belonged to this mycorrhizal state. If no single state were present in more than 60% of species, the family was assigned a "mix" state, to indicate no dominance of any mycorrhizal association.

To evaluate if this criterion biased our results, we analyzed the relationship between mycorrhizal type and diversification rates using different thresholds for mycorrhizal state assignation. For this, we used the 106 families included and remade the analyses but with different thresholds of percentage of species to assign familiar mycorrhizal type. We tested four criteria: 50%, 80% or 100%. 60% is also included here to facilitate comparison.

Table S2. Summary of ANOVAs between mycorrhizal type and diversification rate using different thresholds for mycorrhizal type assignation

Mycorrhizal type assignation	Mycorrhizal type vs Diversification rate (ε=0.0)		Mycorrhizal type vs Diversification rate (ε=0.9)		N° families per group			
	F	p-value	F	p-value	AM	EM	NM	MIX
50%	2.7	0.04	2.3	0.07	81	12	10	3
60%	8.9	2E-05	9.7	1E-05	80	11	8	7
80%	3.9	0.009	3.8	0.01	75	7	4	20
100%	10.8	3E-06	16.8	5E-09	47	3	3	53

Figure S1. Relationship between mycorrhizal type and diversification rate estimated with ε (relative extinction fraction) = 0 (Panels A) and with ε= 0.9 (Panels B), using different thresholds for MIX state assignment (50%, 60%, 80% and 100%). AM: Arbuscular mycorrhiza, EM: Ectomycorrhiza, NM: non-mycorrhizal and MIX (families with no dominance of any specific mycorrhizal association). In each box, the red dots show the mean, the thick black lines show the median, and data is shown with light grey dots.



III. Thresholds for percentage of genera sampled in the phylogeny for the estimation of crown group ages

We calculated diversification rates using crown group ages. Given that crown group ages might be affected by species under-sampling, only the families that had more than 60% of the genera sampled in the phylogeny were included in the analyses.

We tested if the relationship between mycorrhizal type, mycorrhizal diversity index and diversification rates, was conserved using different criteria for diversification rates estimation. Thus, we performed the same analyses but modifying the requirement of % of genera in the phylogeny. We performed the analyses including all families (independent of the % of their genera sampled in the phylogeny, N=157), with a more relaxed criterion (50% of genera had to be sampled in the phylogeny, N=126) with a stricter criterion (80% of genera in the phylogeny) and with the strictest criterion (only families that had 100% of their genera in the phylogeny are included). Results with 60% are shown in the table to comparison.

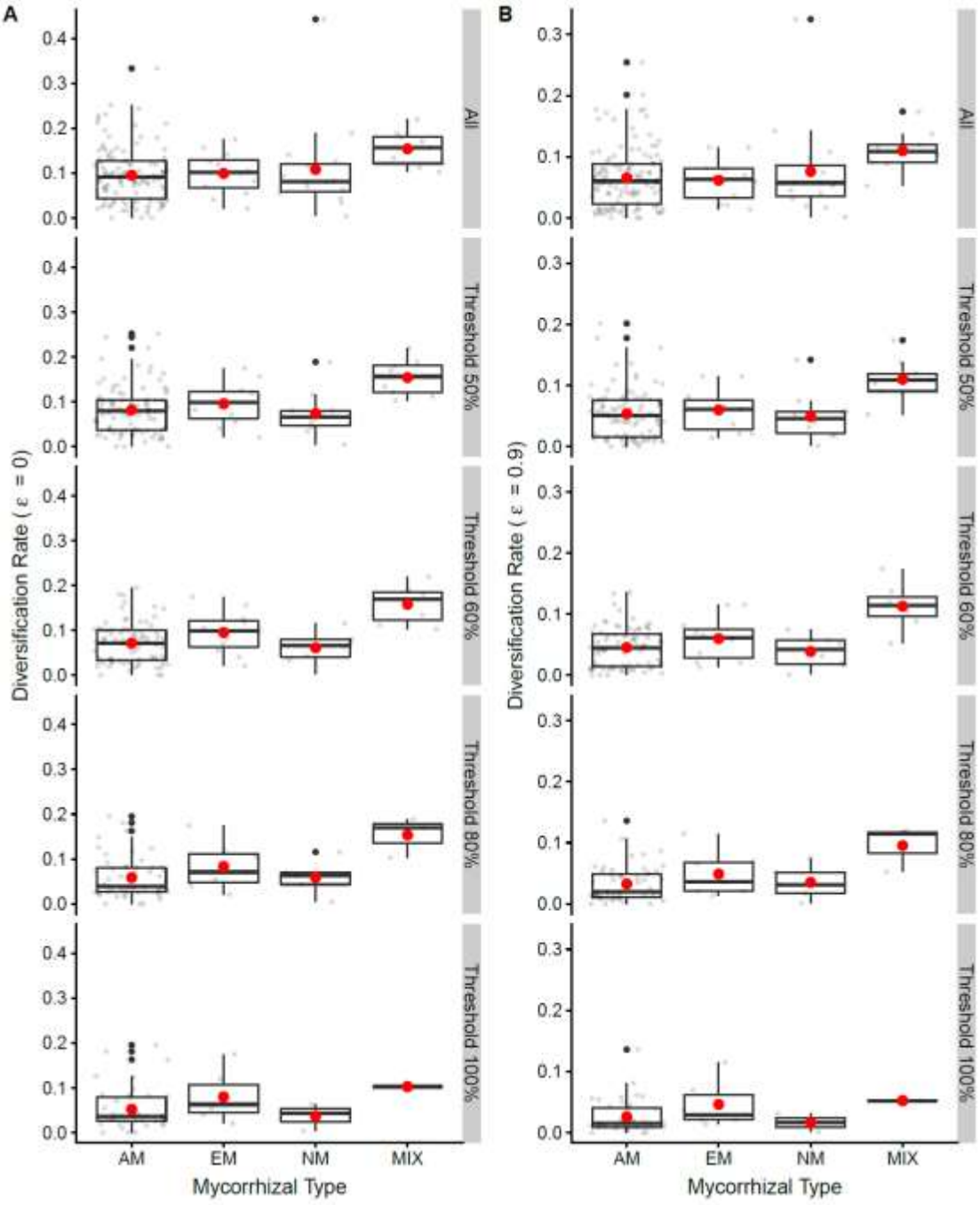
a. Mycorrhizal type vs Diversification rates

*OM and ER are excluded from this analysis

Table S3. Summary of ANOVAs between mycorrhizal type and diversification rate using different thresholds for crown group age estimations.

Threshold for crown group age estimation	Mycorrhizal type vs Diversification rate (ε=0.0)		Mycorrhizal type vs Diversification rate (ε=0.9)		N° families per group				N total
	F	p-value	F	p-value	AM	EM	NM	MIX	
All included	2.4	0.07	2.3	0.08	119	15	12	9	155
50%	5.7	0.001	5.8	0.001	96	11	10	9	126
60%	8.9	3E-05	9.7	1E-05	80	11	8	7	106
80%	4.4	0.007	4.5	0.006	53	7	5	3	68
100%	1.1	0.4	1.3	0.3	41	6	3	1	51

Figure S2. Relationship between mycorrhizal type and diversification rate estimated with ε (relative extinction fraction) = 0 (Panels A) and with ε= 0.9 (Panels B), using different thresholds for crown group age estimation (No filter, N=155; 50%, N=126; 80%, N=86 and 100%, N=51). AM: Arbuscular mycorrhiza, EM: Ectomycorrhiza, NM: non-mycorrhizal and MIX (families with no dominance of any specific mycorrhizal association). In each box, the red dots show the mean, thick black lines show the median, and data is shown with light grey dots.

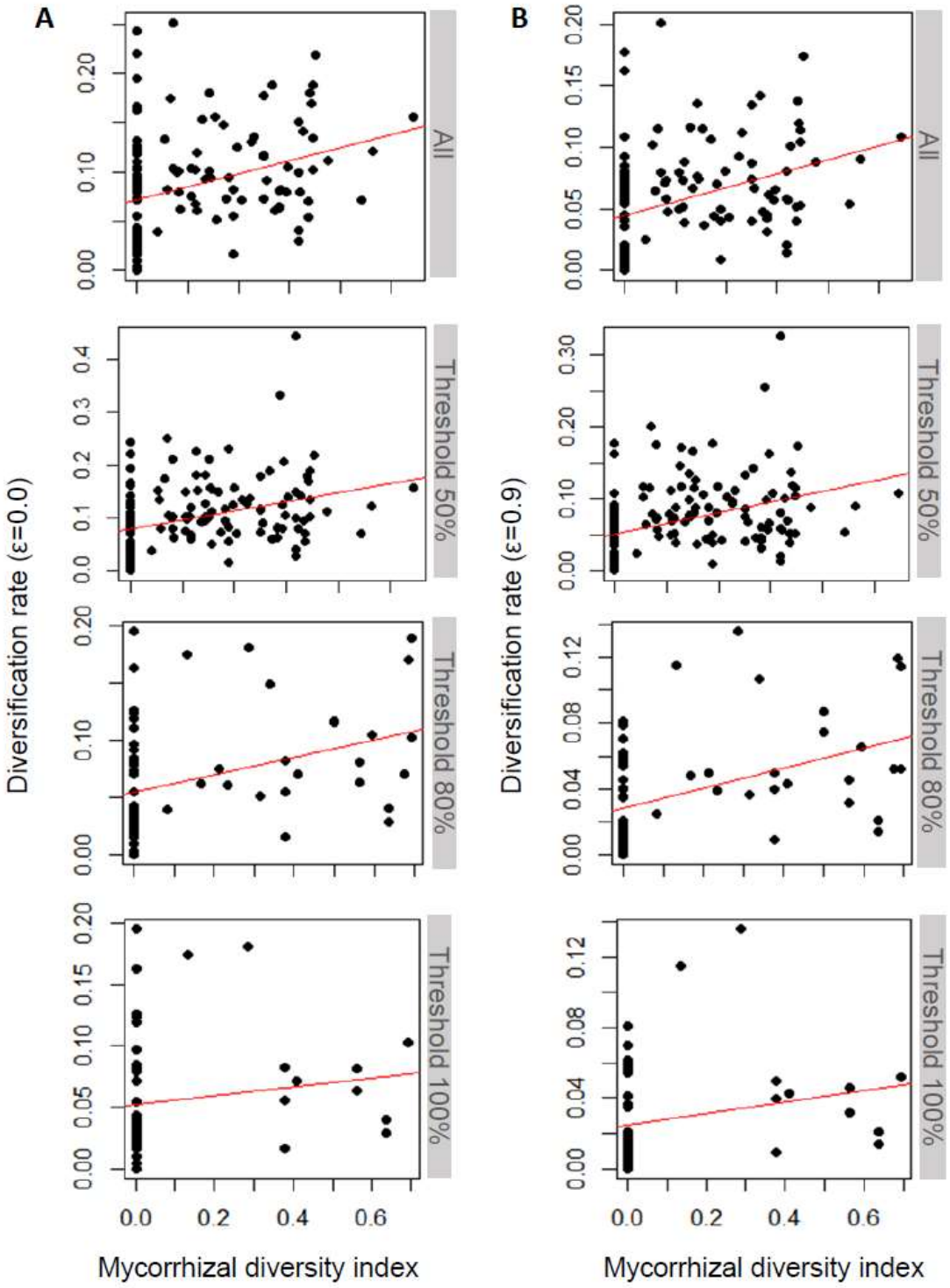


b. Mycorrhizal diversity index vs. Diversification rates

Table S4. Summary of correlations between mycorrhizal diversity index and Diversification rates using different filters for crown group age estimations

Threshold for crown group age estimation	Mycorrhizal diversity index vs Diversification rate (ε=0.0)		Mycorrhizal diversity index vs Diversification rate (ε=0.9)	
	Adjusted R^2	p-value	Adjusted R^2	p-value
All included	0.12	6E-06	0.14	8E-07
50%	0.10	0.0001	0.13	2E-05
60%	0.19	2E-06	0.24	5E-08
80%	0.12	0.003	0.19	0.0001
100%	0.01	0.3	0.03	0.1

Figure S3. Relationship between mycorrhizal diversity index and diversification rate estimated with ε = 0 (Panels A) and with ε= 0.9 (Panels B), using different filters for crown group age estimation (No filter, N=155; 50%, N=126; 80%, N=86 and 100%, N=51).



IV. Phylogenetic signal

Table S5. Phylogenetic signal of mycorrhizal traits and diversification rates

Trait	Blomberg´s K	Pagel´s Lambda
Diversification rate (ε=0.0)	0,72*	0,37*
Diversification rate (ε=0.5)	0,71*	0,36*
Diversification rate (ε=0.9)	0,62*	0.26
Mycorrhizal diversity index	0.41	4,68 e-5

Mycorrhizal type	N° of families	D value
AM	132	- 0.31*
NM	15	0.11*
EM	17	0.57
MIX	9	-0.74*

V. Results of ANOVA and phylogenetic ANOVA

Table S6. Results of ANOVA and phylogenetic ANOVA between mycorrhizal type and diversification rate.

Model	ANOVA		Phylogenetic ANOVA	
	F	p-value	F	p-value
Mycorrhizal type vs Diversification rate (ε=0.0)	8.9	2.6 e-5	8.9	0.002
Mycorrhizal type vs Diversification rate (ε=0.5)	9.2	1.9 e-5	9.2	0.001
Mycorrhizal type vs Diversification rate (ε=0.9)	9.7	1.1 e-5	9.7	0.001

Table S7. *A posteriori* analysis of ANOVA and phylogenetic ANOVA

ANOVA	Corrected P-values of Tukey multiple comparisons of means					
	MIX-AM	MIX-EM	MIX-NM	AM-EM	AM-NM	EM-NM
Mycorrhizal type vs Diversification rate (ε=0.0)	0.0000206	0.02	0.0003	0.36	0.92	0.36
Mycorrhizal type vs Diversification rate (ε=0.5)	0.000014	0.02	0.0003	0.37	0.92	0.37
Mycorrhizal type vs Diversification rate (ε=0.9)	0.0000057	0.006	0.0002	0.52	0.95	0.51

ANOVA	Corrected P-values of Tukey multiple comparisons of means					
	MIX-AM	MIX-EM	MIX-NM	AM-EM	AM-NM	EM-NM
Mycorrhizal type vs Diversification rate (ε=0.0)	0.0000206	0.02	0.0003	0.36	0.92	0.36
Mycorrhizal type vs Diversification rate (ε=0.5)	0.000014	0.02	0.0003	0.37	0.92	0.37
Mycorrhizal type vs Diversification rate (ε=0.9)	0.0000057	0.006	0.0002	0.52	0.95	0.51

Phylogenetic ANOVA	Pairwise corrected P-values					
	MIX-AM	MIX-EM	MIX-NM	AM-EM	AM-NM	EM-NM
Mycorrhizal type vs Diversification rate (ε=0.0)	0.01	0.10	0.01	0.64	0.64	0.64
Mycorrhizal type vs Diversification rate (ε=0.5)	0.01	0.08	0.01	0.70	0.70	0.70
Mycorrhizal type vs Diversification rate (ε=0.9)	0.01	0.02	0.01	0.88	0.88	0.88

Figure S4. Relationship between the proportion any specific mycorrhizal type in the family and their diversification rate estimated with ε= 0 (Panels A) and with ε= 0.9 (Panels B). AM: Arbuscular mycorrhiza, EM: Ectomycorrhiza, NM: non-mycorrhizal. r^2 of each model is shown in each graph.

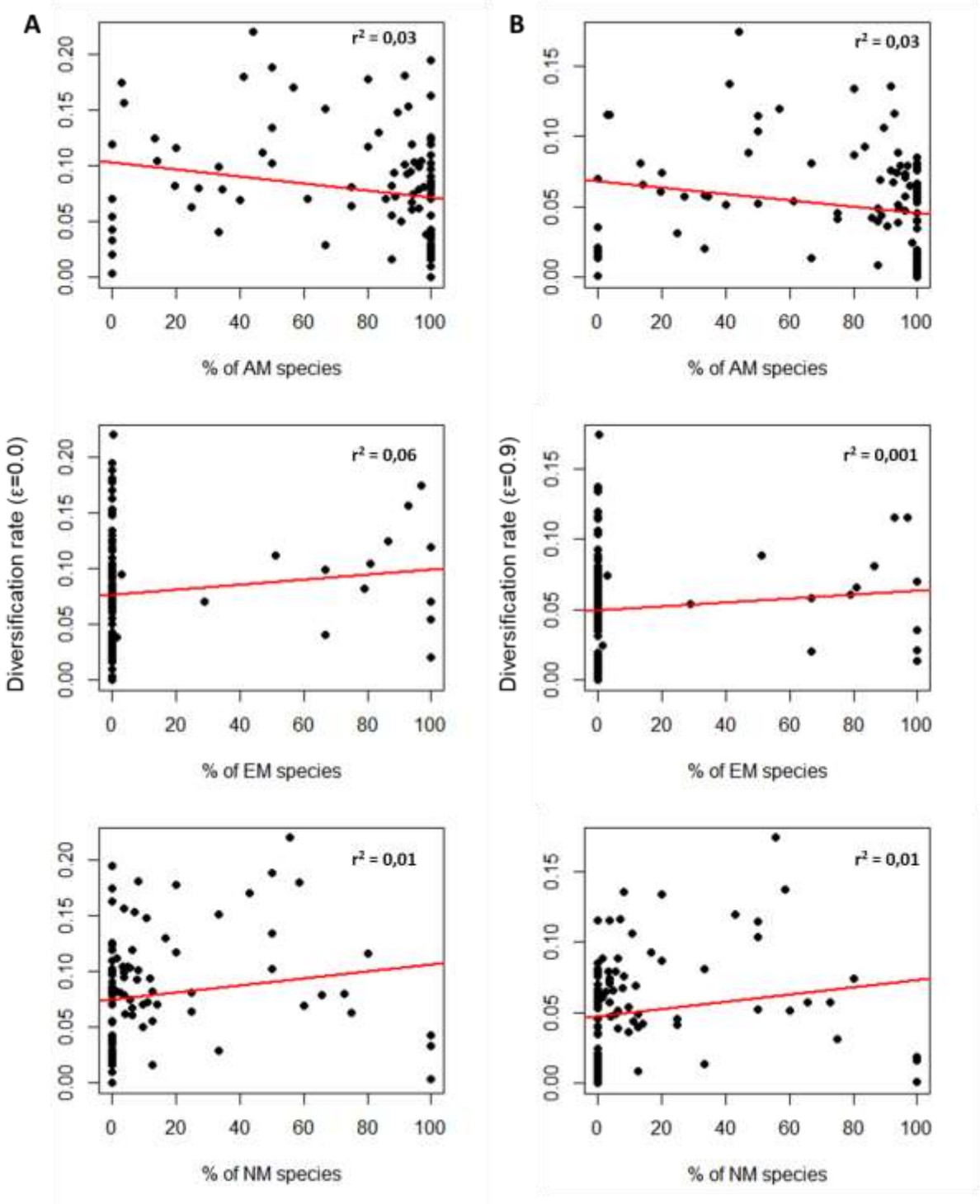
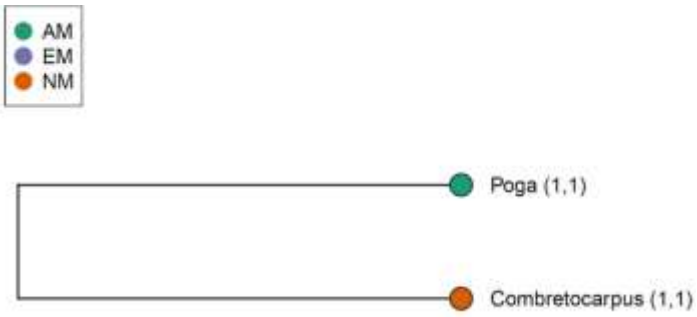
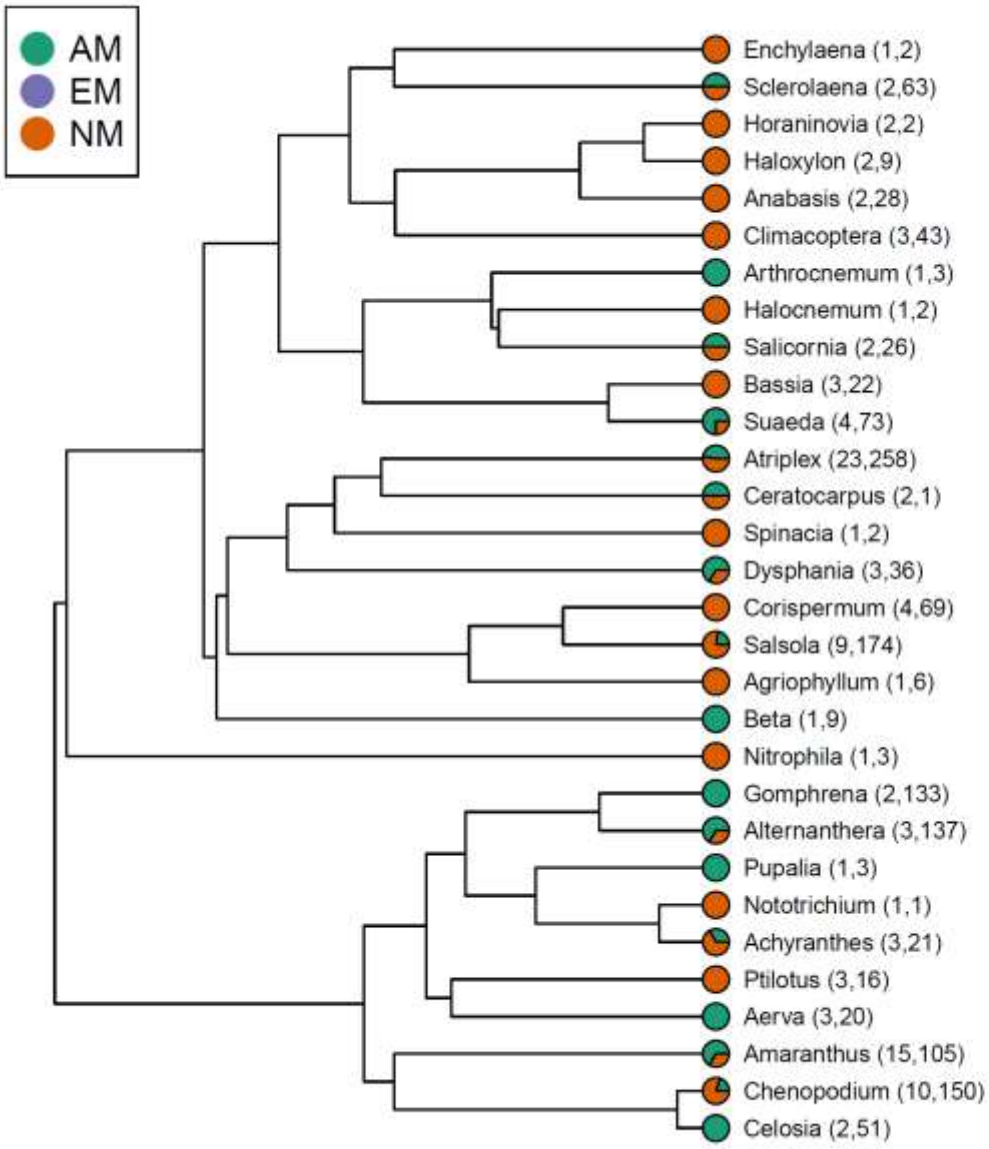


Figure S5. Genus-level phylogenies of each mixed family. Pie charts shows the proportion of species with arbuscular mycorrhizas (AM; green), ectomycorrhizas (EM; blue) and without mycorrhizas (NM; orange) within each genus. Parentheses next each genus show two numbers: the first is the number of species of the genus present in the mycorrhizal states database and the second is the total number of species of the genus, according to TheplantList.org. Phylogenies were obtained by extracting each clade from the Vascular plants phylogeny (Zanne *et al*., 2014) and pruning the genera with no mycorrhizal data.

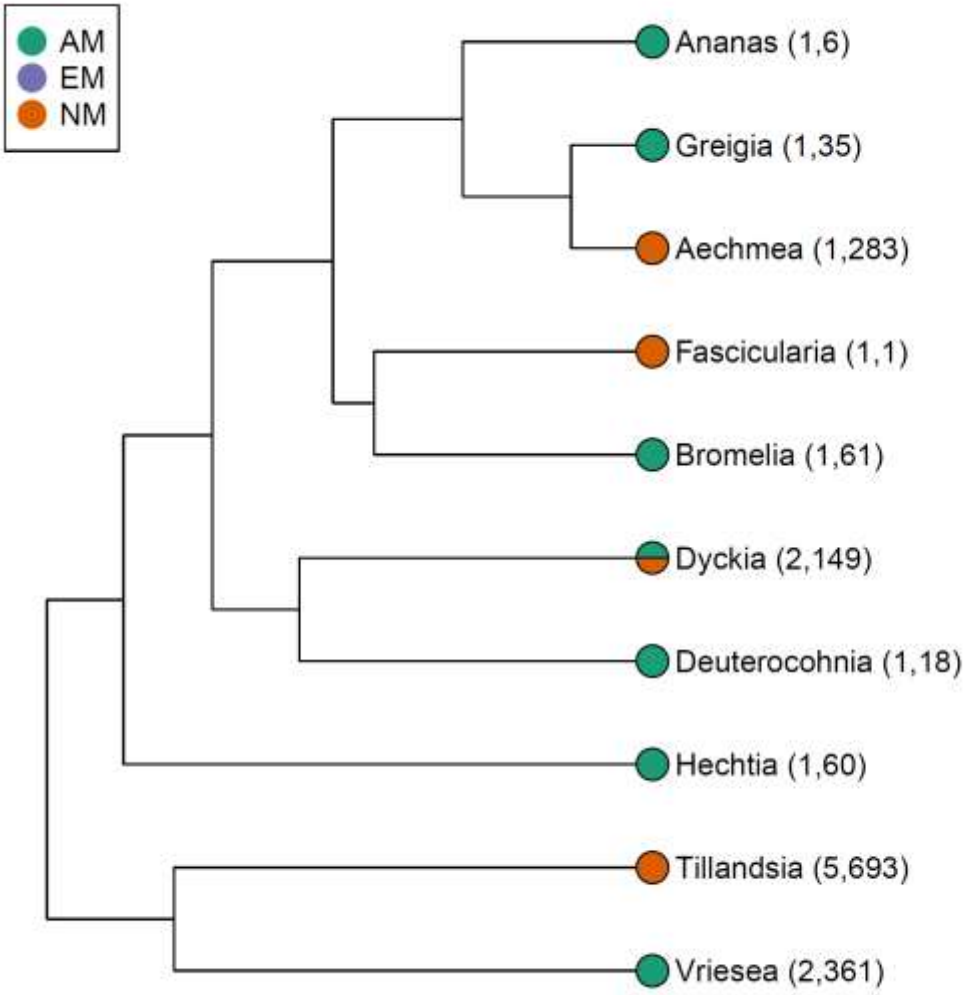
a) Anisophyllaceae



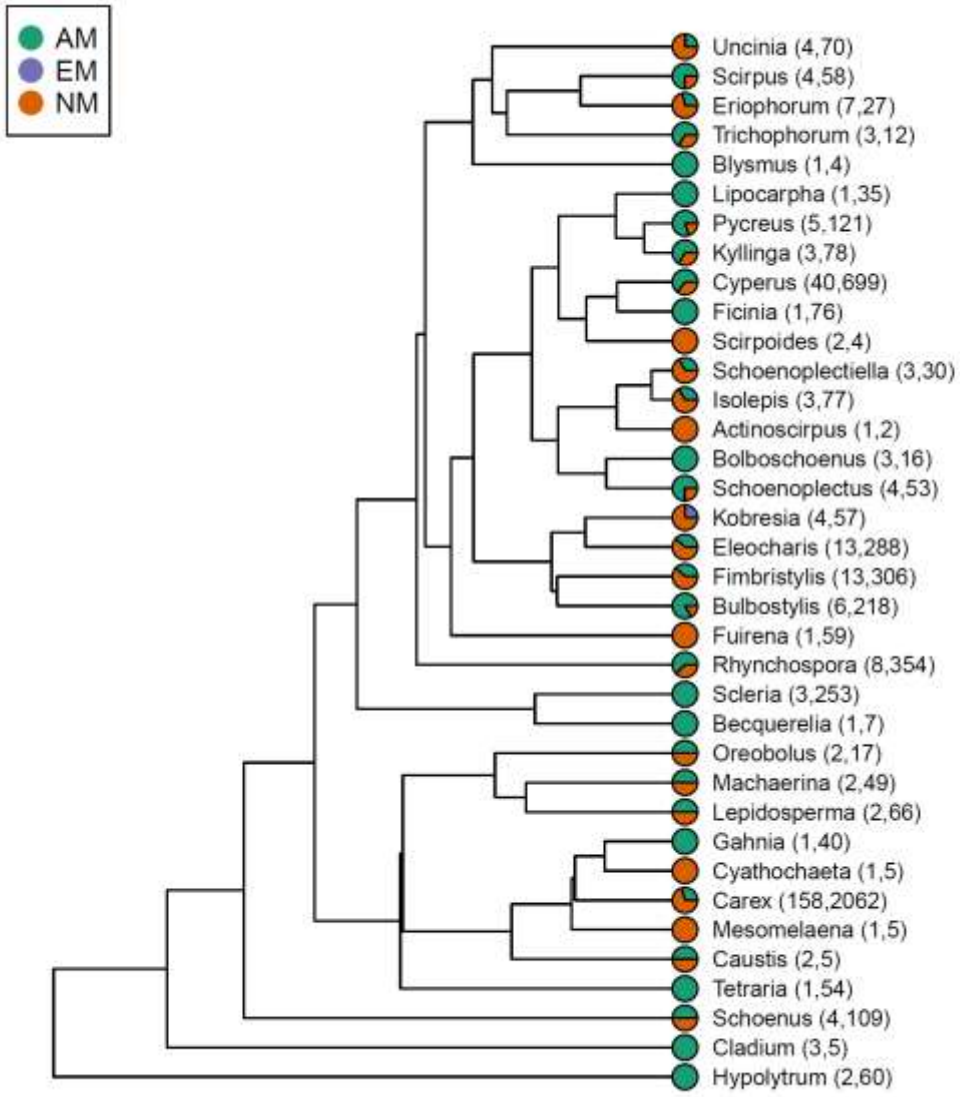
b) Amaranthaceae



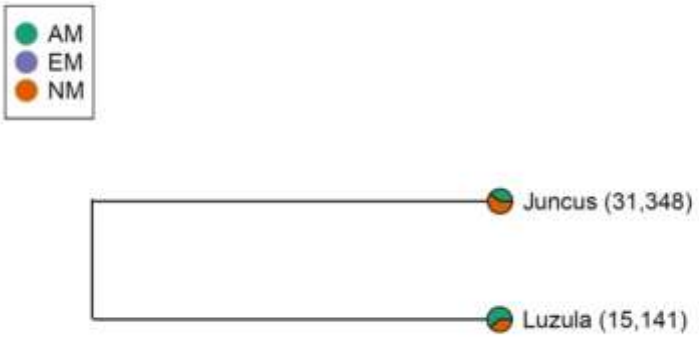
c) Bromeliaceae



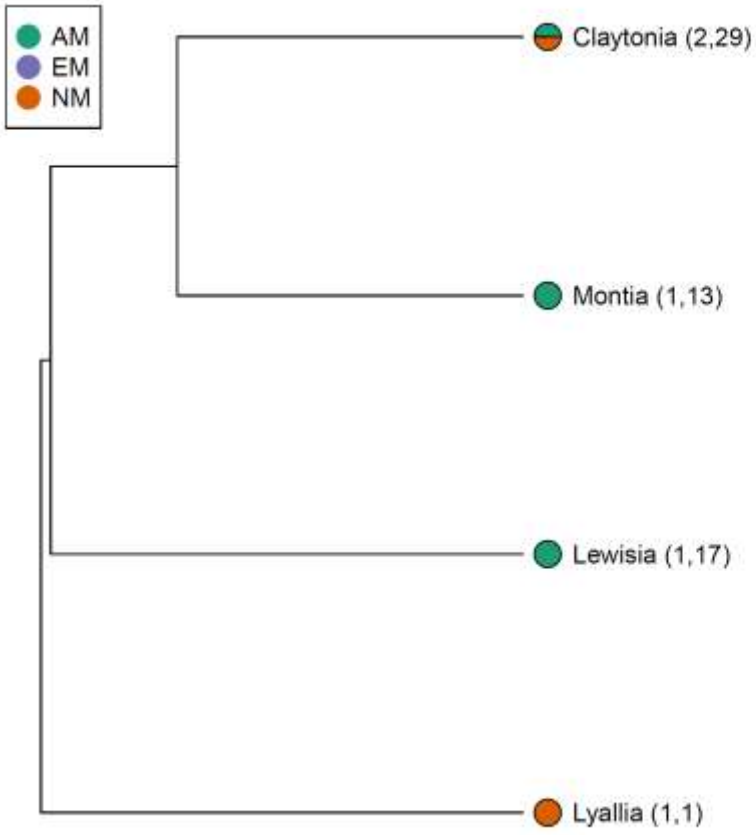
d) Cyperaceae



e) Juncaceae



f) Montiaceae



g) Myrtaceae

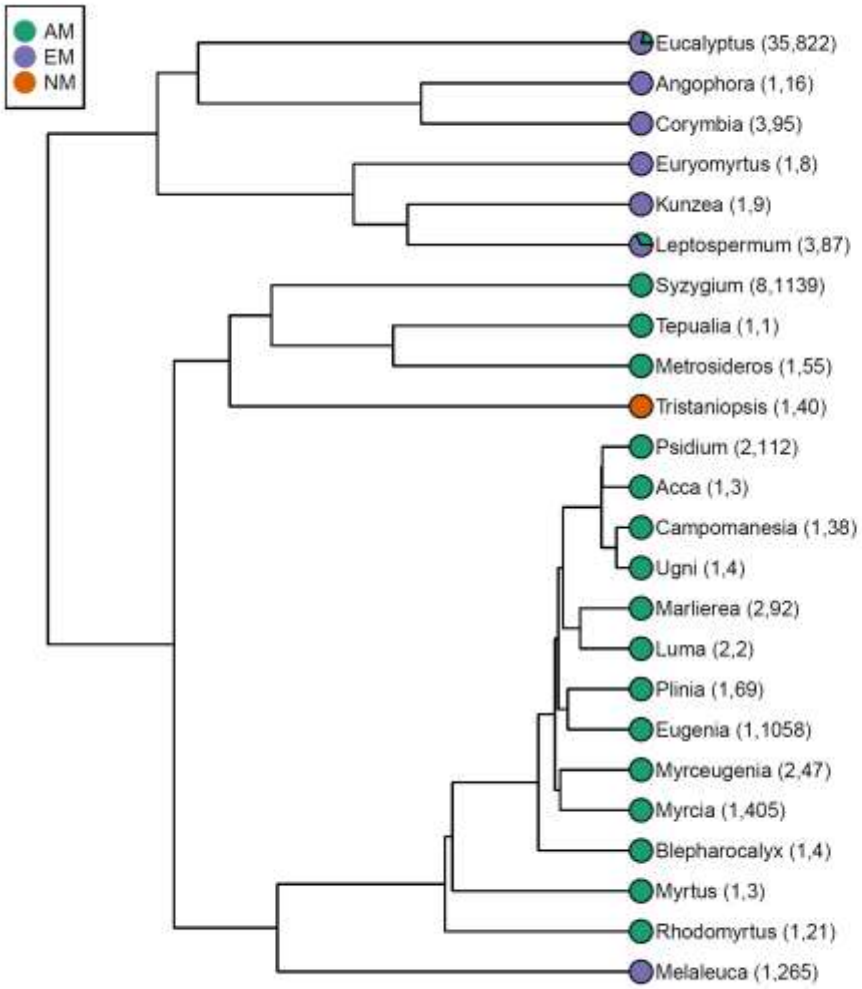

AM
EM
NM
Eucalyptus (35,822)
Angophora (1,16)
Corymbia (3,95)
Euryomyrtus (1,8)
Kunzea (1,9)
Leptospermum (3,87)
Syzygium (8,1139)
Tepualia (1,1)
Metrosideros (1,55)
Tristaniopsis (1,40)
Psidium (2,112)
Acca (1,3)
Campomanesia (1,38)
Ugni (1,4)
Marlierea (2,92)
Luma (2,2)
Plinia (1,69)
Eugenia (1,1058)
Myrceugenia (2,47)
Myrcia (1,405)
Blepharocalyx (1,4)
Myrtus (1,3)
Rhodomyrtus (1,21)
Melaleuca (1,265)

Table S8.

Family	% AM species	% EM species	% NM species	Family Mycorrhizal Type	Diversification rate (ϵ=0.0)	Diversification rate (ϵ=0.9)	Mycorrhizal diversity Index
Acoraceae	100	0	0	AM	0.000	0.000	0.000
Adoxaceae	93.75	0	6.25	AM	0.061	0.039	0.234
Alismataceae	88.89	0	11.11	AM	0.072	0.044	0.349
Altingiaceae	100	0	0	AM	0.126	0.054	0.000
Amaranthaceae	41.03	0	58.97	MIX	0.180	0.137	0.678
Amaryllidaceae	96.72	0	3.28	AM	0.104	0.080	0.144
Anacardiaceae	96.30	0	3.70	AM	0.099	0.071	0.158
Anarthriaceae	100	0	0	AM	0.036	0.013	0.000
Anisophylleaceae	50	0	50	MIX	0.102	0.052	0.693
Annonaceae	92.86	0	7.14	AM	0.153	0.117	0.257
Araliaceae	91.67	0	8.33	AM	0.101	0.076	0.287
Araucariaceae	100	0	0	AM	0.018	0.009	0.000
Arecaceae	96.15	0	3.85	AM	0.062	0.048	0.168
Asparagaceae	94.55	0	5.45	AM	0.103	0.080	0.212
Atherospermataceae	100	0	0	AM	0.028	0.012	0.000
Begoniaceae	91.67	0	8.33	AM	0.181	0.136	0.287
Berberidaceae	90.48	0	9.52	AM	0.051	0.037	0.314
Betulaceae	2.94	97.06	0	EM	0.175	0.115	0.133
Biebersteiniaceae	100	0	0	AM	0.038	0.012	0.000
Bixaceae	100	0	0	AM	0.041	0.019	0.000
Bromeliaceae	50	0	50	MIX	0.134	0.104	0.693
Burseraceae	100	0	0	AM	0.119	0.085	0.000
Canellaceae	100	0	0	AM	0.079	0.035	0.000
Cannabaceae	87.50	0	12.50	AM	0.082	0.049	0.377
Cannaceae	0	0	100	NM	0.043	0.017	0.000
Caprifoliaceae	92.31	0	7.69	AM	0.092	0.067	0.271
Carlemanniaceae	100	0	0	AM	0.020	0.006	0.000
Caryocaraceae	100	0	0	AM	0.124	0.058	0.000
Casuarinaceae	27.27	72.73	0	EM	0.119	0.070	0.000
Celastraceae	88.24	0	11.76	AM	0.094	0.070	0.362
Ceratophyllaceae	0	0	100	NM	0.004	0.001	0.000
Cistaceae	12.50	87.50	0	EM	0.125	0.081	0.393
Commelinaceae	30	0	70	NM	0.080	0.058	0.586
Convolvulaceae	93.75	0	6.25	AM	0.119	0.089	0.234
Coriariaceae	100	0	0	AM	0.195	0.081	0.000
Cucurbitaceae	100	0	0	AM	0.090	0.066	0.000
Cupressaceae	98.39	1.61	0	AM	0.039	0.025	0.084

Cycadaceae	100	0	0	AM	0.019	0.009	0.000
Cyperaceae	43.08	0.31	55.97	MIX	0.220	0.174	0.705
Diapensiaceae	0	100	0	EM	0.055	0.021	0.000
Dioscoreaceae	87.50	0	12.50	AM	0.055	0.039	0.377
Dipterocarpaceae	14.29	80.95	4.76	EM	0.105	0.066	0.594
Elaeagnaceae	85.71	0	14.29	AM	0.071	0.043	0.410
Ephedraceae	87.50	0	12.50	AM	0.016	0.009	0.377
Escalloniaceae	100	0	0	AM	0.036	0.019	0.000
Euphorbiaceae	97.47	0	2.53	AM	0.081	0.065	0.118
Fagaceae	3.45	93.10	3.45	EM	0.156	0.115	0.307
Geraniaceae	100	0	0	AM	0.082	0.060	0.000
Gnetaceae	33.33	66.67	0	EM	0.040	0.021	0.637
Goodeniaceae	100	0	0	AM	0.091	0.062	0.000
Griseliniaceae	100	0	0	AM	0.025	0.008	0.000
Hydrangeaceae	94.44	0	5.56	AM	0.075	0.049	0.215
Iridaceae	100	0	0	AM	0.087	0.066	0.000
Irvingiaceae	100	0	0	AM	0.163	0.061	0.000
Juglandaceae	33.33	66.67	0	EM	0.099	0.058	0.637
Juncaceae	54.35	0	45.65	MIX	0.170	0.119	0.684
Juncaginaceae	25	0	75	NM	0.063	0.031	0.562
Krameriaceae	100	0	0	AM	0.036	0.017	0.000
Lardizabalaceae	100	0	0	AM	0.030	0.015	0.000
Lauraceae	100	0	0	AM	0.083	0.064	0.000
Liliaceae	96.15	0	3.85	AM	0.101	0.073	0.163
Lythraceae	83.33	0	16.67	AM	0.130	0.093	0.451
Magnoliaceae	100	0	0	AM	0.081	0.054	0.000
Melanthiaceae	100	0	0	AM	0.071	0.045	0.000
Meliaceae	100	0	0	AM	0.110	0.079	0.000
Melianthaceae	100	0	0	AM	0.026	0.011	0.000
Montiaceae	50	0	50	MIX	0.188	0.114	0.693
Moraceae	80	0	20	AM	0.117	0.087	0.500
Moringaceae	100	0	0	AM	0.038	0.015	0.000
Myricaceae	100	0	0	AM	0.074	0.040	0.000
Myrtaceae	47.95	50.68	1.37	MIX	0.112	0.089	0.755
Nepenthaceae	100	0	0	AM	0.026	0.008	0.000
Nitrariaceae	100	0	0	AM	0.028	0.011	0.000
Nothofagaceae	0	100	0	EM	0.071	0.036	0.000
Nymphaeaceae	0	0	100	NM	0.033	0.019	0.000
Oleaceae	89.29	0	10.71	AM	0.148	0.107	0.340
Pandaceae	100	0	0	AM	0.036	0.015	0.000
Petrosaviaceae	100	0	0	AM	0.010	0.003	0.000
Philesiaceae	100	0	0	AM	0.000	0.000	0.000

Phyllanthaceae	61.29	29.03	9.68	AM	0.071	0.054	0.885
Pinaceae	0	100	0	EM	0.020	0.014	0.000
Platanaceae	100	0	0	AM	0.024	0.009	0.000
Podocarpaceae	100	0	0	AM	0.027	0.017	0.000
Potamogetonaceae	20	0	80	NM	0.116	0.075	0.500
Proteaceae	40	0	60	NM	0.070	0.052	0.673
Ranunculaceae	93.86	0	6.14	AM	0.067	0.051	0.231
Resedaceae	66.67	0	33.33	AM	0.151	0.081	0.637
Rhamnaceae	96.15	0	3.85	AM	0.079	0.058	0.163
Rosaceae	93.01	3.31	3.68	AM	0.095	0.074	0.290
Salicaceae	19.74	78.95	1.32	EM	0.082	0.061	0.564
Sapindaceae	100	0	0	AM	0.102	0.077	0.000
Saxifragaceae	34.48	0	65.52	NM	0.079	0.057	0.644
Schisandraceae	100	0	0	AM	0.034	0.019	0.000
Scrophulariaceae	80	0	20	AM	0.178	0.134	0.500
Staphyleaceae	66.67	0	33.33	AM	0.084	0.041	0.000
Stegnospermataceae	100	0	0	AM	0.016	0.005	0.000
Strelitziaceae	100	0	0	AM	0.043	0.014	0.000
Surianaceae	100	0	0	AM	0.030	0.010	0.000
Tamaricaceae	83.33	0	0	AM	0.097	0.056	0.000
Taxaceae	100	0	0	AM	0.019	0.009	0.000
Tetrameristaceae	100	0	0	AM	0.000	0.000	0.000
Tofieldiaceae	66.67	0	33.33	AM	0.029	0.014	0.637
Typhaceae	75	0	25	AM	0.081	0.045	0.562
Vitaceae	100	0	0	AM	0.055	0.041	0.000
Xanthorrhoeaceae	100	0	0	AM	0.102	0.076	0.000
Zygophyllaceae	75	0	25	AM	0.064	0.042	0.562

Capítulo II: The influence of soil nutrients on root fungal community associated with the terrestrial orchid *Bipinnula fimbriata*

Title: The influence of soil nutrients on root fungal community associated with the terrestrial orchid *Bipinnula fimbriata*

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ABSTRACT

Little is known about the factors that influence root-associated fungal communities in Orchidaceae. The limited evidence suggests that soil nutrients may modulate the association with orchid mycorrhizal fungi (OMF), but their influence on non-mycorrhizal fungi remains unexplored. In a previous regional study, we observed a relationship between nutrients availability and mycorrhizal associations in *Bipinnula fimbriata*. To explore if nutrient availability affected in a different way the mycorrhizal and the non-mycorrhizal fungi associated with *B. fimbriata*, we conducted a closer investigation in a large population of *B. fimbriata* using next-generation sequencing. Additionally, we tested the effect of nutrient addition on fungal communities and mycorrhizal colonization. We found a high diversity of fungal associates in roots of *Bipinnula fimbriata* and a strong spatial structure of root fungal communities. Soil P was negatively related to the abundance of OMF but not to the abundance of non-mycorrhizal fungi. After fertilization, increments in soil P negatively affected mycorrhizal colonization; however, it had no effect on OMF richness or composition. The abundance and richness of pathogens were negatively related to mycorrhizal colonization, and then after fertilization, the decrease in mycorrhizal colonization was related to an increase in pathogen richness. Our results suggest that OMF is affected by soil conditions in a different way than the non-mycorrhizal fungal guilds. Also, that this orchid responds to fertilization by altering mycorrhizal colonization rather than shifting OMF partners, and that the influence of nutrients on OMF community is coupled with indirect effects on the whole fungal community.

Keywords: Orchid mycorrhiza, fungal root community, fungal guilds, orchid fertilization, soil nutrients.

INTRODUCTION

Fungal communities that colonize plant roots can be highly diverse (Vandenkoornhuyse *et al*. 2002). Almost all plant species have fungal root associates (Rodriguez, 2009; Hardoim *et al* 2015) and they have been crucial for the evolution of plants (Pyrosinsky & Malloch, 1975; Selosse & de Tary, 1989). These fungal symbionts can greatly influence plant fitness (Brundrett, 2006; Smith and Read 2008), plant interactions, and long-term plant dominance (Molina *et al*., 1992, Van der Heijden *et al*., 2003). Great focus has been placed on mycorrhizal fungi, which can increase plant nutrient uptake (Marschner & Dell, 1994) and help species to tolerate new environmental conditions (McCormick *et al.*, 2006), but non-mycorrhizal endophytes also can confer fitness benefits to host plants, including tolerance to heat, disease, and drought (Rodriguez & Redman, 2008). The functions of fungal root communities could be highly influenced by the diversity and composition of the fungal species involved (Alzarani *et al*., 2018), therefore one main issue is to understand how environmental factors determine the diversity and composition of root fungal communities (including mycorrhizal and non-mycorrhizal fungi).

Root fungal communities are greatly influenced by abiotic and biotic factors (David *et al*., 2016), including host plants (Roy *et al*., 2013), climate (Tedersoo *et al*., 2012) and soil conditions (Huggins *et al*., 2014; Van Geel *et al*., 2017; Boeraeve *et al*., 2018; Blaalid *et al*., 2014). Particularly, soil nutrients play a major role in regulating diversity and composition of fungal root associates (Peay *et al*., 2009; Polme *et al*., 2013; Yao *et al*., 2013), they can also affect mycorrhizal colonization, which tends to decrease under nitrogen (N) and phosphorus (P) fertilization (Treseder, 2004; Blechem & Alexander, 2012; Balzergue *et al*., 2013).

Less is known about the role of soil nutrients on fungal root associates in Orchidaceae. Orchids commonly form mycorrhizal associations with fungi belonging to three families (Tulasnellaceae, Sebacinaceae, and Ceratobasidiaceae) of the basidiomycetes (Dearnaley *et al*., 2012); and in addition to orchid mycorrhizal fungi (OMF), a large diversity of endophytic fungi can be found in association orchid roots (Bayman & Otero, 2006). The limited evidence suggests that soil nutrients may play a key role in orchid mycorrhizas. Bunch *et al.* (2013), for example, showed that the composition of mycorrhizal fungi varies among populations of the orchid *Cypripedium acaule* in association with soil pH and percentage organic matter, C and N, whereas Mujica *et al*. (2016) found a significant relationship between soil phosphorus (P) and nitrogen (N) and the composition and diversity of mycorrhizal fungi associated with two species of *Bipinnula.* Nevertheless, the effect of soil nutrients on diversity and composition of non-mycorrhizal fungal associates remains unknown. It has been suggested that the soil environment may impact mycorrhizal associations and other types of fungal associations in similar ways (Bunch *et al.*, 2013), given that soil conditions may act as an environmental filter for the whole fungal community. Contrary, soil nutrients may differentially affect the mycorrhizal and non-mycorrhizal fungi, which could result from different tolerances to nutrient availabilities among different functional groups or from plant selection of mycorrhizal partners modulated by soil nutrients.

In previous work, we conducted a correlation field study in B*ipinnula fimbriata*, a terrestrial orchid endemic to Central Chile (Mujica *et al*., 2016), including seven population of this species which cover over 600 km. We found a positive relationship between soil P availability and mycorrhizal colonization and OMF richness; and a negative correlation between soil nitrate and OMF phylogenetic diversity. However, as it was a correlational study and the

diversity of non-mycorrhizal fungi remained unevaluated, experimental studies are needed to assess the role of soil nutrients on OMF communities and to elucidate their effect on the non-mycorrhizal associates. In this study, we conducted a closer investigation in a large population of *Bipinnula fimbriata* using next-generation sequencing to assess (1) if nutrient availability was related in a different way with the mycorrhizal and the non-mycorrhizal fungi associated with *B. fimbriata* roots, (2) if nutrient addition influenced OMF communities and mycorrhizal colonization as expected from the regional correlation study and (3) if nutrient addition influenced the non-mycorrhizal fungi associated with *B. fimbriata*. To do this, we first evaluated the relationships between soil nutrients and the diversity of the whole fungal community associated with *Bipinnula fimbriata* (A). Then, we conducted the fertilization and evaluated how nutrient addition affected the community of mycorrhizal and non-mycorrhizal species associated with *Bipinnula fimbriata* (B).

MATERIALS AND METHODS

Orchid species and study site

Bipinnula Comm. ex Juss. (subtribu Chloraeinae, Orchidoideae) is a genus of terrestrial, photosynthetic orchids endemic to southern South America. It comprises a separate group of five species endemic to Chile (Novoa *et al*., 2006; Cisternas *et al*., 2012). *Bipinnula fimbriata* (Poepp.) Johnst. is the most frequent of these five species; it is distributed in lowland (<500 m) coastal areas from 29 to 35°S (Novoa *et al*., 2006), preferably on sandy stabilized soils, in open sites exposed to sunlight and marine breezes (Elortegui & Novoa, 2009). The flowering season spans from middle winter to late spring (approx. July to November).

The study was conducted in a large population of *Bipinnula fimbriata* located at the “La Cruz” hill, which is situated near to the town Zapallar, in the Coast of central Chile (32°33 S, 71°28

W; 30 m above sea level; Fig. 1). The climate of this site is Mediterranean with a mean annual temperature of 14.2° and mean annual precipitation of 384 mm (Luebert & Pliscoff, 2012) and the soil type is clayey and stony. The vegetation of the hill is coastal Mediterranean and consists mainly of sclerophyllous shrubs such as *Baccharis macraei* and *Puya chilensis*, perennial herbs as *Bahia ambrosioides* and *Happlopapus foliosus* and annual species such as *Pasithea coerulea* and *Alstroemeria pulchra*. The population of *Bipinnula fimbriata* covers the entire hill, forming four main subpopulations in four zones: North, South, East, and West.

Sampling and fertilization experiment

20 vegetation plots of 50 x 50 cm were established in the study site. The plots had between three and five adult plants of *Bipinnula fimbriata*, were situated with at least 1 m distance between them, and covered the entire orchid population, including the four main zones. During the flowering season of 2015, three root samples were collected from each plot, on three different plants, resulting in 60 root samples. To avoid unnecessary plant damage, each root sample consisted of only three root pieces per plant, leaving the rhizome intact, so that the plant could grow further in the next season. Collected roots were individually labeled and kept cold during transport to the laboratory, where they were processed for further analysis. For soil nutrient analyses, one mixed soil sample was extracted in each plot, obtained from the soil that surrounded orchid roots. Soils samples were dried at 60°C for 24 hours, sieved and then analyzed for P Olsen, percent N, percent C and pH in the Laboratory of Biogeochemistry at the Pontificia Universidad Católica de Chile. After sampling, two nutrient treatments were established with 10 plots each. The treatment assignation to plots was randomized but ensuring that each treatment was present in the four zones. The treatments were Phosphorus (P), which consisted in 10 gr of SPT in 0.5 L of distilled water and a Control treatment (C)

which was only 0.5 L of distilled water, with no addition of nutrients. Treatments were applied with a wash bottle covering all the surface of each plot with the fertilization solution. The same sampling procedure conducted the first year was conducted one year after fertilization during the flowering season (spring 2016).

S*amples preparation*

Roots pieces were washed under tap water to remove soil and dirt and sterilized as follows: samples were placed for 1 min in 1% sodium hypochlorite and then three times consecutively for 3 min in sterile distilled water. Orchid mycorrhizal fungi form pelotons in root cortex cells. In *Bipinnula*, as in many other orchid species, groups of pelotons can be noted on the washed root surfaces as spots ranging in color from light yellow to dark brown. For each root piece, the level of colonization by mycorrhizal fungi was quantified as the fraction of the root surface covered by spots (produced by the presence of pelotons). Roots with the verified presence of pelotons were cut into 3mm sections, placed in sterile 2mL tubes and stored at -20 °C until DNA extraction. DNA was extracted using a cetyltrimethylammonium bromide (CTAB) method modified from Doyle & Doyle (1990).

Metabarcoding analysis

To investigate the fungi associated with roots of the studied plants we used two pairs of primers for each sample to amplify the ITS2 region of the ribosomal DNA, namely ITS86F-ITS4 and ITS3-ITS4OF (Table S1). PCR profile was set up as follows: 95 °C for 7 min, followed by 30 cycles at 94 °C for 30 s, 59.2 °C for 40 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min. The obtained PCR products were then amplified in a second PCR using unique tags to identify each individual sample. PCR was run with similar conditions except for the annealing temperature of 60.4 °C. The obtained samples were purified with NucleoMag©

NGS Clean-up and subsequently, as one pooled amplicon library, were sent for sequencing with Ion Torrent PGM (Life Technologies, Carlsbad, USA). The sequenced reads were processed following a procedure similar to the one described in Schneider-Maunoury *et al*. (2018). Briefly, reads were first demultiplexed and split based on the present tags, subsequently screened for the presence of the primers using Cutadapt version 1.15 (Martin, 2011) and trimmed. Next, we generated a reference database of OTUs (operational taxonomic units) using the QIIME package, version 1.9.1 (Caporaso *et al*., 2010). First, we clustered the reads with the SWARM algorithm (Mahé *et al*., 2014). After removing singletons, for each picked OTU we next selected one representative sequence, as well as removed chimeras incorporating the UCHIME software (Edgar *et al*., 2011) to map against the reference dataset for ITS chimera detection (Nilsson *et al*., 2015) version 7.2 (release 2017-06-28) obtained from the UNITE database (Nilsson *et al*., 2019). Next, we filtered and trimmed the original sequencing reads based on the presence of ITS86F or ITS4 primers using Cutadapt software. Using BLASTN (Altschul *et al*., 1990) we clustered the filtered reads into OTUs by searching against the OTU reference database generated in the previous steps. Assignment of taxonomy for each OTU was finally recovered by comparing their representative sequences against the reference fungal database UNITE version 7.2 (release 2017-12-01) (Nilsson *et al*., 2019) using the BLAST algorithm.

Fungal guilds

Fungal OTUs were classified into the following guilds: OMF, patotroph, saprotrophs, and symbiotroph. Orchid mycorrhizal fungi were assigned following Dearnaley *et al.,* (2012) and the non-mycorrhizal fungal OTUs were classified into three different trophic modes following the classification of FunGuild v1.0 (http://www.stbates.org/guilds/app.php). The guilds were

assigned according to the nutrient acquisition mode: (1) pathotroph: nutrient acquisition by harming host cells (including phagotrophs); (2) symbiotroph: by exchanging resources with host cells; and (3) saprotroph: by breaking down dead host cells (Nguyen *et al*., 2016).

Statistical analyses

We first analyzed the data of the pre-fertilization samples. We tested the effects of zone and soil nutrients on composition, relative abundance (measured as the proportion of reads in the sample) and richness of each fungal guild and on the level of colonization by OMF. To assess the effects on the composition of the whole root fungal community, fungal guild composition was calculated as the relative abundance of each fungal guild in each plot. The dissimilarity between plots was calculated using Bray–Curtis distances (Bray & Curtis, 1957) and then we performed a variance analysis of these distances, using a multivariate permutation test (Adonis test) implemented in the Vegan package of R (Oksanen *et al*., 2013). We included zone, soil nutrients and the interactions between these factors. The effect of Zone and nutrients on plot ordination was illustrated by a non-metric multidimensional scaling (NMDS) on Bray–Curtis distances. To further explore the effect of the same factors on the composition of each fungal guild, we calculated the composition of each guild separately. To assess the effect of zone and soil nutrients on the relative abundance and OTU richness of each fungal guild, we used generalized linear models (GLMs). Each response variable was modeled separately, testing the following components: Zone, nutrients and the interactions between Zone and soil nutrients. In this way, we tested whether the effect of soil nutrients on mycorrhizal fungi differed among Zones. Models were built using a bidirectional stepwise selection procedure, starting with a full model and alternately omitting and re-introducing one model component at each step (Pearce & Ferrier, 2000). Models were selected according to the lowest values of the Akaike

and Bayesian information criteria (AIC and BIC, respectively). The GLM deviance was estimated as the goodness of fit. Significant relationships among variables were described using partial residual plots of the most likely model as judged by the AIC. To further explore the relationship between mycorrhizal fungi and non-mycorrhizal fungi with an independent measure of OMF abundance (rather than relative abundances), we evaluated the relationship between non-mycorrhizal guilds and mycorrhizal colonization. Finally, to test the effect of fertilization, the same analyses on OMF relative abundance, diversity and composition were performed, but with samples obtained in the year after the fertilization data and including fertilization treatment as a factor.

RESULTS

Fungal community found in Bipinnula fimbriata *roots*

A total of 667 fungal OTUs were retrieved from 118 plants sampled, including fungi from 33 classes belonging to 10 phyla (Table 1). The orders with the highest number of OTUs were Pleosporales (66), Chaetothyriales (40), Hypocreales (38) and Agaricales (35). OTUs were assigned to the guilds Orchid mycorrhizal fungi (OMF), pathotroph, saprotroph or symbiotroph (Fig. 1). There were 11 OTUs that could be considered as putative species of OMF, from which three OTUs were related to members of the Tulasnellaceae, including two species of *Tulasnella*, *T. calospora* and *T. asymetrica*, and one unidentified Tulasnellaceae; three to the Ceratobasidiaceae, including an unidentified species from the genus *Ceratobasidium*, *Thanatephorus cucumeris* and one unidentified Ceratobasidiaceae; and four to the Sebacinales, including *Serendipita vermifera*, two unidentified species of the families Sebacinaceae and Serendipitaceae and one OTU unidentified of the Sebacinales. Most of the plants had OMF fungi in their roots (86.4%), with the OTU Ceratobasidiaceae as the most

frequent (83%, Fig. 2), however, there were some plants without OMF fungi. The OTU Pezizaceae was found in 85.5% of plants, including in roots of those plants where OMF was not detected. Pezizaceae has been suggested to be mycorrhizal in Orchids (Dearnaley *et al*., 2012), but given that its mycorrhizal function remains unclear, it was considered as a separate guild for composition analyses. For the rest of the non-mycorrhizal species (656 OTUs), it was possible to assign the putative trophic mode to 533 of them using FunGuild database. These OTUs were assigned into pathotroph (119), saprotroph (188 OTUs) or symbiotroph (53) guilds, or to the mixed guilds saprotroph-symbiotroph (23), pathotroph-saprotroph (70), pathotroph-symbiotroph (26), pathotroph-saprotroph-symbiotroph (49). Among the most frequent OTUs in each guild were the pathotrophs *Ilyonectria mors-panacis* and *Rhexocercosporidium panacis*, the saprotophs OTUs *Filobasidium stepposum* and *Cladophialophora* sp, and the symbiotrophs *Rhodotorula glutinis* and *Cladosporium sphaerospermum* (Fig. 2). Also, a large number of ectomycorrhizal genera were found, including *Scleroderma*, *Cortinarius*, *Cenococcum*, and *Tomentellopsis*.

Fungal guild composition

Fungal guild composition was significantly affected by Zone and soil P, but not by the other soil nutrients (Fig. 3A), as shown by the Adonis analysis (Table 2). While North and East zones were more abundant in OMF, the West Zone is equally abundant in OMF and Pezizaceae, and the South is more abundant in pathotrophs with a very low abundance of OMF (Fig. 3B). Accordingly, GLM analyses on the relative abundance of each guild showed that some guilds were influenced by soil P, also showing that each fungal guild responds differentially to nutrients and Zone. While OMF relative abundance was influenced by Zone, soil P and soil % C (AIC null model = 1.5, AIC best model =-30.3, R^2=0.85), showing a

negative relationship with soil P and a positive relationship with soil % C (Fig.4A and B); the relative abundance of pathotrophs was significantly explained by Zone, soil P and soil pH (AIC null model = -12.35, AIC best model =-24.8, R^2=0.6), showing a positive relationship with pH, and contrary to OMF, a positive relationship with soil P. The relative abundance of the saprotroph fungi was also affected by Zone and soil pH (AIC null model = -50, AIC best model =-57.69, R^2=0.51), and the relative abundance of symbiotroph and Pezizaceae sp. showed no differences among zones and no effect of soil nutrients.

When the composition of each guild was analyzed separately, it also showed an effect of Zone. For the case of OMF composition, NMDS showed a clear differentiation between zones (Fig. 3C), which was statistically supported by ADONIS analysis (Zone effect: F=3.12, p=0.002). Composition of the other three guilds also showed an effect of Zone, which according to the Adonis analysis was significant in the symbiotrophs and nearly significant in the other two guilds (Zone effect in symbiotrophs F=3.7, p=0.02; pathotroph F=1.2, p=0.06; saprotroph F=1.8, p=0.08).

Fungal richness

Mycorrhizal richness varied considerably among plants, which had between zero and 6 putative OMF OTUs. The best model explaining this variation included Zone (AIC null model= 76.9, AIC best model =67.4, R^2=0.45), the zones North and South had the lowest richness while the zones East and West had the highest (Fig. 4D). There was no effect of soil nutrients and Zone on the richness of the other three fungal guilds.

Mycorrhizal colonization and non-mycorrhizal fungi

The opposite relationship that showed pathotroph and OMF relative abundances with soil P suggested a possible negative relationship between both guilds. To further explore this

relationship with an independent measure of OMF abundance (rather than relative abundances), we evaluated the relationship between non-mycorrhizal guilds and mycorrhizal colonization. The relative abundance and the richness of pathotrophs were both significantly affected by the interaction between Zone and Mycorrhizal colonization (Abundance model: R^2=0.77; Richness model: R^2=0.56). Therefore, these relationships were analyzed separately by zone. There was a negative relationship between colonization and pathogen relative abundance in the South zone (R^2=0.79, p=0.01), a negative but slightly significant relationship in the West zone (R^2=0.6, p=0.07) and no significant relationship in North and East zones (Fig. 5A). Regarding richness, the relationship between pathotroph richness and mycorrhizal colonization was stronger in the South zone (R^2=0.85, p=0.005) and East zone (R^2=0.85, p=0.05), nearly significant in North (R^2=0.61, p=0.07) and no relationship was observed within plots of the West zone (Fig. 5B). The richness of saprotrophs also showed a significant but lower relationship with mycorrhizal colonization (AIC null model =142.2, AIC best model = 136, R^2=0.3) and there was no effect of mycorrhizal colonization on symbiotroph fungi.

Effects of fertilization

Soil P Olsen was significantly higher in P plots than in Control plots (F=42.7 P=3.8e-6), however, there was considerable variation within each treatment and when analyzing plots separately, 52% of them showed an increase in soil P after fertilization. There was no significant effect of Treatment on OMF relative abundance, richness and composition. Also, there was no direct effect of Treatments on Mycorrhizal colonization, however, when considering plots that presented an increment of soil P after fertilization, there was a significant relationship between the increase in soil P and decrease on Mycorrhizal colonization (R^2=0.55 p=0.02) (Fig. 6A). There was no direct effect of Treatment on the

richness and relative abundance of pathotrophs, saprotrophs, and symbiotrophs. However, when considering plots where mycorrhizal colonization decreased, there was a significant relationship between the increase in pathotroph richness and decrease in mycorrhizal colonization (R^2=0.21, p=0.05; Fig. 6B).

DISCUSSION

Diversity of fungal community in Bipinnula fimbriata *roots*

To our knowledge, this is the first study that assesses fungal community associated with a terrestrial orchid in South America using metagenomics. This study contributes to filling a big gap of knowledge of fungal root associates of terrestrial orchids in this region (Jacquemyn *et al*., 2017) showing a high diversity of fungal species, including mycorrhizal and non-mycorrhizal fungi. Across all samples, we found 11 putative OTUs of orchid mycorrhizal fungi, belonging to the families Tulasnellaceae, Ceratobasidiaceae, and fungi from Sebacinales. This diversity is similar to what has been reported to *Bipinnula fimbriata* across different populations (Steinfort *et al*., 2010; Herrera *et al*., 2017; Mujica *et al*., 2016), with the exception of Sebacinales, which were not detected by those studies. The same lack of Sebacinales has been observed for other terrestrial orchid species from southern South America that have used fungal culturing techniques (Flores-Aguilera *et al*., 2019; Herrera *et al*., 2019; Fracchia *et al*., 2014). This difference suggests that techniques of culturing fungi from roots could have had overlooked the presence of Sebacinales, but this needs further investigation.

Plants ranged from zero to 6 Orchid mycorrhizal fungal OTUs. There were 16 plants with no OMF OTUs, however, it is interesting to notice that 11 of them did present OTUs from Pezizaceae, similar to what Waterman *et al.* (2011) observed in some South African orchid

species. There was a high frequency of the OTU "Pezizaceae sp." in the roots of *Bipinnula fimbriata*, being present in 98 from the 118 plants sampled. Putative Peziza spp. were also observed associated in other populations of *B. fimbriata* and *B. plumosa* (Mujica *et al*., 2016). Indeed, Pezizomycetes are usually found associated with orchid roots (Stark *et al*., 2009; Jiang *et al*., 2011; Ma *et al*., 2015) and it has been suggested as a possible mycorrhizal partner of orchids (Dearnelay *et al.*, 2012). Further studies are needed to assess the role of these species, considering its high presence in orchid roots.

We found a high diversity of non-mycorrhizal fungi, although the ecological roles of these fungi in orchids are largely unknown (Jiang *et al*., 2011) and fungi may have more complex niches than previously thought (Selosse *et al*., 2018), the assignation of trophic guilds can give us a clue of the function of these fungal species on roots of *B. fimbriata*. We detected the presence of pathogenic fungi as *Ilyonectria mors-panacis, Rhexocercosporidium panacis* and *Fusarium oxysporum. I. mors-panacis* and *R. panacis* are highly pathogenic in ginseng (*Panax quinquefolius*) (Reedeler *et al*., 2002; Farh *et al*., 2017) but we did not find any report of these species infecting orchid roots. On the other hand, *F. oxysporum* has been demonstrated to cause root rot and other symptoms in several commercial orchid species (Kim *et al*., 2002; Lee *et al*., 2002; Swett & Uchida, 2015). Among the saprophytic taxa observed, some of them such as *Humicola* sp, *Acremonium* spp., and *Trichoderma,* have been reported in other orchid species (Bayman & Otero, 2006). Similarly, some of the symbiotroph taxa that we detected, such as *Hypoxylon* and *Colletotrichum,* have been shown to promote growth in plantlets of the orchid *Rhynchostylis retusa* (Shah *et al*., 2018). Nevertheless, the ecological role of most orchid roots endophytes remains largely unknown (Jiang *et al*., 2011) and deserve further investigation.

Fungal guild composition

Fungal guild composition highly varied among the four zones of *B. fimbriata* population, which reflects a strong spatial structure of OMF communities, also related to soil P. While North and East zones were more abundant in OMF, the West Zone was equally abundant in OMF and Pezizaceae (although potential OMF), and the South was more abundant in pathotroph, followed by saprotrophs and with a very low abundance of OMF (Fig 3). This variation may be explained partly by differences on soil nutrients among zones, given that the South zone had significantly lower soil P ($F= 5.03$, $p=0.005$) than the others. This zone has also lower -but not significant- mycorrhizal colonization. This may suggest that soil P could be limiting OMF abundance and colonization. When analyzing across zones, however, we found a negative relationship between soil P and relative abundance of OMF (Fig. 4A). At lower levels of soil nutrients (N or P) plants tend to allocate more carbon to mycorrhizal partners (Johnson *et al*., 2003) to maximize the nutrient uptake, thus possibly increasing abundance of mycorrhizal fungi within roots. Changes in C allocation as a function of nutrient availability has not been studied in orchid mycorrhizas, but it is likely to occur, as photosynthetic orchids supply C to mycorrhizal fungi (Cameron *et al*., 2006) and receive inorganic P from them (Cameron *et al*., 2007). The negative relationship between soil nutrients and root fungal abundance is not expected to occur in non-mycorrhizal fungi, given that plants do not obtain nutrients from these fungi. Accordingly, there was no negative correlation between the relative abundance of non-mycorrhizal fungi and soil P.

The effect of soil P on orchid mycorrhizal association may differ depending on the spatial scale observed. Differences among zones suggest that soil P could be limiting OMF abundance or colonization, which agrees with the positive relationship between soil P and

richness and mycorrhizal colonization observed at the regional scale in *Bipinnula fimbriata* (Mujica *et al*., 2016). However, when we investigated the variation among plots, soil P negatively correlated with OMF abundance. At the regional scale, soil P may be acting as an environmental filter, affecting the pool of OMF available in soils, but at the local scale, soil P affects the association between plants and OMF, modulating the abundance of OMF and colonization within roots. Multilevel investigations are needed to test these hypotheses (Lilleskov & Parrent, 2007), including the screening of OMF in soils and the effect of soil nutrients at different spatial scales.

In addition to soil P, the soil percent of C also appears to affect the relative abundance of OMF. There was a positive relationship between C and the relative abundance of OMF (Fig. 4B), which could be explained by the saprophytic nutritional mode of OMF fungi (Dearnaley *et al*., 2012). Although soil percent of C includes total organic and inorganic carbon, a higher percent of C could be related to higher organic carbon, indicating higher availability of nutritional resources for OMF. In a similar way, Bunch *et al*. (2013) found that orchid populations growing in environments with moderate to higher levels of C, N and organic matter, had a higher diversity of potential fungal associates.

Fungal richness

Contrary to what was observed with relative abundance, OMF richness varied only as a function of Zone and was not affected by soil P. This result suggests that while OMF abundance varies across the natural variation of P, this variation is not coupled with variation in OMF richness. Similarly, the richness of the other fungal guilds showed no relationship with soil nutrients but also no differences among zones.

Mycorrhizal colonization and pathotroph fungi

We found that mycorrhizal colonization was negatively related to richness and relative abundance of pathotrophs, suggesting a potential role of orchid mycorrhizal fungi on defense against pathogens. The relationship between pathogen abundance and mycorrhizal colonization has not been reported before in orchid mycorrhizas, however defense role of mycorrhizal colonization has been frequently reported in other mycorrhizal associations (Marx, 1972; Newsham *et al*., 1994; Azcón-Aguilar & Barea, 1996). Recently, Herrera *et al*., (2018) found that mycorrhizal root segments of *Bipinnula fimbriata* stimulated protein synthesis related to pathogen control, which could partially explain our observations. Future research, including experimental studies, are required to test the role of mycorrhizal colonization in pathotroph defense in orchids, to know how widespread this relationship is within Orchidaceae, and to understand the underlying mechanisms.

Fertilization experiment

Addition of P had no significant effect on richness abundance or composition of OMF. However, it negatively affected the mycorrhizal colonization, as the increase of P significantly decreased mycorrhizal colonization. This effect has been reported in fertilization experiments in arbuscular and ectomycorrhizas (Baum & Makeschin, 2000; Treseder & Vitousek 2001) and it is probably explained by the less carbon allocation to mycorrhizal fungi under nutrient enrichment (Johnson *et al*., 2003). Remarkably, the changes in mycorrhizal colonization were not related to significant changes in mycorrhizal richness or composition, suggesting that, at this temporal and spatial scale, plants reacted to P addition through changes in mycorrhizal colonization rather than in diversity associated. Switching fungal partner under environmental changes has been observed in *Goodyera pubescens* (McCormick *et al*., 2006), however, fungal switching only seemed to occur under extreme conditions, during which the initial fungus

likely died, and it was also associated with substantial mortality (McCormick *et al*., 2006). Thus, switching fungi appeared to be the last recourse to face extreme conditions rather than a response to minor environmental fluctuations. Then, it is likely that the addition of P was not extreme enough to trigger a switching on the fungal partner, but it was enough to produce changes in the level of colonization. Finally, while P addition apparently had no direct effect on non-mycorrhizal diversity, our results suggest that it may have an indirect effect on pathotrophs trough negatively affecting mycorrhizal colonization. Given that mycorrhizal colonization was negatively correlated with abundance and richness of root pathotrophs, it was expected that a decrease in mycorrhizal colonization could increase the abundance of these fungi. This result suggests that P fertilization can influence the whole fungal community within roots, by affecting the level of mycorrhizal colonization.

Conclusions

We found a high diversity of fungal associates in roots of *Bipinnula fimbriata*, much more to what was known by isolating fungal cultures from roots. We also observed for the first time the presence of fungi from Sebacinales, whose potential function as mycorrhizal fungi requires further investigation. Our results showed that soil P was negatively related to the abundance of OMF, but not with the non-mycorrhizal endophytes. Also when P was experimentally added, it had a negative effect on mycorrhizal colonization. We also observed a negative relationship between mycorrhizal colonization and richness and abundance of pathogens, and that increments of soil P indirectly caused an increment of pathogens richness in *B. fimbriata* roots. This observation opens new questions in the study of orchid mycorrhizas and suggests that in addition to seed germination, development and nutrient exchange, orchid mycorrhizal fungi may play a role in defense against pathogens. Finally, our results support the hypothesis that

different mycorrhizal and non-mycorrhizal fungi respond differentially to soil nutrient availabilities. However, the effect of soil P on OMF communities can have an indirect influence on the non-mycorrhizal fungal community.

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FIGURE LEGENDS

Figure 1. The geographic location of the study site "Cerro La Cruz" (A) Regional map indicating the location of the town Zapallar, where Cerro la Cruz is situated. (B) Picture of *Bipinnula fimbriata* growing in the study site. (C) Frequency of each fungal guild and the most frequent families of each guild associated with *B. fimbriata*.

Figure 2. Incidence of the most frequent OTUs in each fungal grid found in the roots of *Bipinnula fimbriata*. *The role of fungi from the family Pezizaceae as a mycorrhizal associate of Orchidaceae is still unknown, but the OTU Pezizaceae is included to show its high incidence in *Bipinnula fimbriata* plants.

Figure 3. Non-metric multidimensional scaling (NMDS) ordination plots based on the composition of (A) the whole fungal community associated with *Bipinnula fimbriata* and (B) putative mycorrhizal fungal species. Explanatory variables found to be significant ($P>0.05$) in the Adonis test are represented as a vector (soil P) or by colors (Zone). (C) Pie charts showing the abundance of each fungal guild in each zone of the *B. fimbriata* population in Cerro La Cruz. The black line represents 100 m.

Figure 4. Relationships between soil nutrients and zone and abundance and richness of Orchid Mycorrhizal fungi (OMF) in the pre-fertilization conditions. OMF abundance as a function of P Olsen (A), Soil % of C (B) and Zone (C). OMF richness as a function of Zone (D)

Figure 5. Relationship between mycorrhizal colonization and (A) relative abundance and (B) richness of pathotroph fungi. There was a significant effect of the interaction between Zone and Mycorrhizal colonization, thus relationships are plot separately by zone. Dotted lines show significant relationships.

Figure 6. Relationship between Mycorrhizal colonization, P Olsen and Pathotroph richness. (A) Increase in P Olsen and changes in Mycorrhizal colonization and (B) decrease in Mycorrhizal colonization and changes in pathotroph richness.

TABLE 1. Number of operational taxonomic units (OTU) by fungal class found in roots of *Bipinnula fimbriata*

Phyllum	Class	N° of OTUs
Ascomycota	Dothideomycetes	114
Ascomycota	Sordariomycetes	90
Ascomycota	Eurotiomycetes	76
Ascomycota	Leotiomycetes	44
Ascomycota	Saccharomycetes	22
Ascomycota	Lecanoromycetes	13
Ascomycota	Pezizomycetes	8
Ascomycota	Orbiliomycetes	6
Ascomycota	Taphrinomycetes	3
Ascomycota	Arthoniomycetes	1
Basidiomycota	Agaricomycetes	95
Basidiomycota	Tremellomycetes	57
Basidiomycota	Microbotryomycetes	23
Basidiomycota	Cystobasidiomycetes	14
Basidiomycota	Exobasidiomycetes	13
Basidiomycota	Ustilaginomycetes	6
Basidiomycota	Agaricostilbomycetes	5
Basidiomycota	Pucciniomycetes	3
Basidiomycota	Tritirachiomycetes	1
Basidiomycota	Moniliellomycetes	1
Chytridiomycota	Spizellomycetes	4
Chytridiomycota	Rhizophlyctidomycetes	2
Chytridiomycota	Chytridiomycetes	1
Chytridiomycota	Rhizophydiomycetes	1
Glomeromycota	Glomeromycetes	5
Glomeromycota	Paraglomeromycetes	2
Glomeromycota	Archaeosporomycetes	1
Mucoromycota	Mucoromycetes	15
Mucoromycota	Umbelopsidomycetes	7
Entomophthoromycota	Basidiobolomycetes	1
Monoblepharomycota	Monoblepharidomycetes	1
Mortierellomycota	Mortierellomycetes	11
Olpidiomycota	Olpidiomycetes	1

TABLE 2. Effects of zone and soil nutrients on fungal guild composition of fungal communities associated with roots of *Bipinnula fimbriata*

Factor	d.f.	F	R^2	P-value
Zone	3	6.59	0.54	0.00
P	1	3.07	0.08	0.05
N	1	1.30	0.04	0.29
C	1	0.32	0.01	0.79
pH	1	2.02	0.06	0.15

FIGURES

Figure 1.

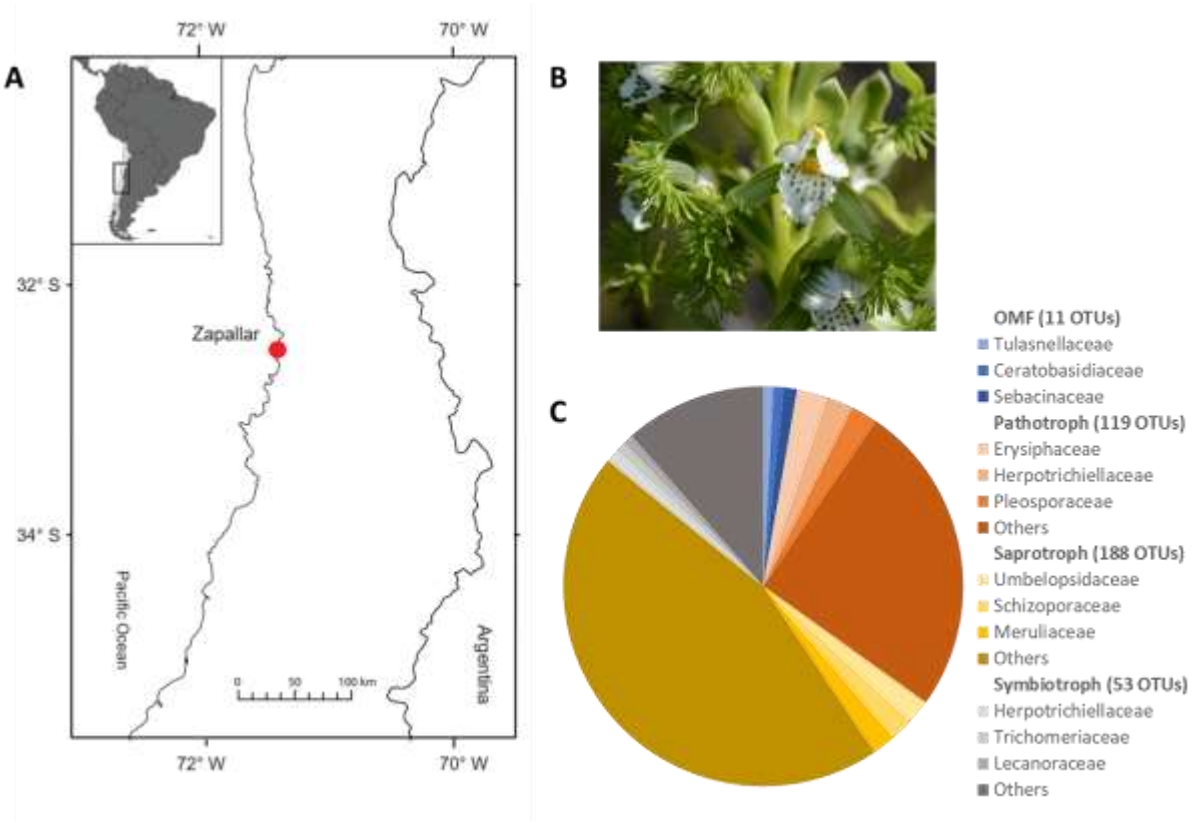


Figure 2.

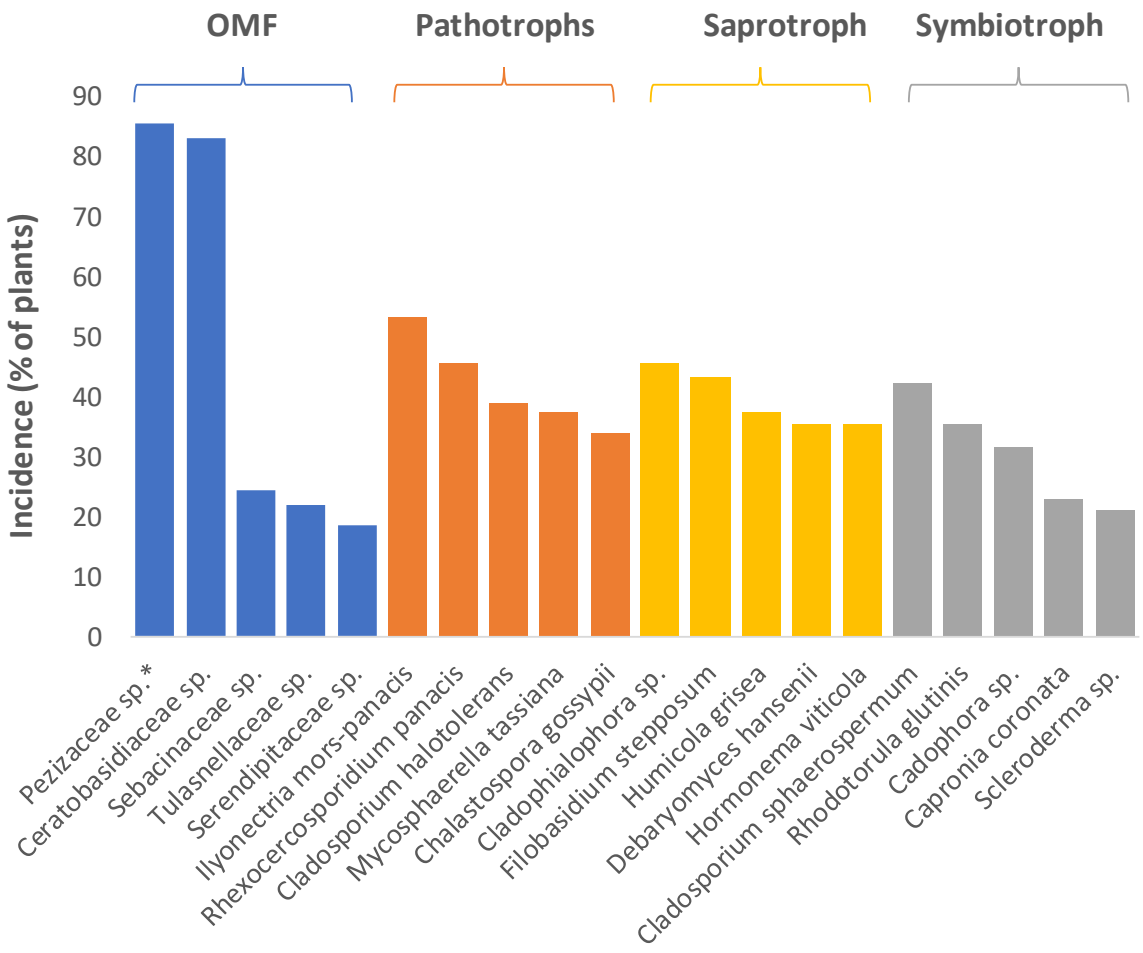

OMF
Pathotrophs
Saprotroph
Symbiotroph
Incidence (% of plants)
90
80
70
60
50
40
30
20
10
0
Pezizaceae sp.*
Ceratobasidiaceae sp.
Sebacinaceae sp.
Tulasnellaceae sp.
Serendipitaceae sp.
Ilyonectria mors-panacis
Rhexocercosporidium panacis
Cladosporium halotolerans
Mycosphaerella tassiana
Chalastospora gossypii
Cladophialophora sp.
Filobasidium stepposum
Humicola grisea
Debaryomyces hansenii
Hormonema viticola
Cladosporium sphaerospermum
Rhodotorula glutinis
Cadophora sp.
Capronia coronata
Scleroderma sp.

Figure 3.

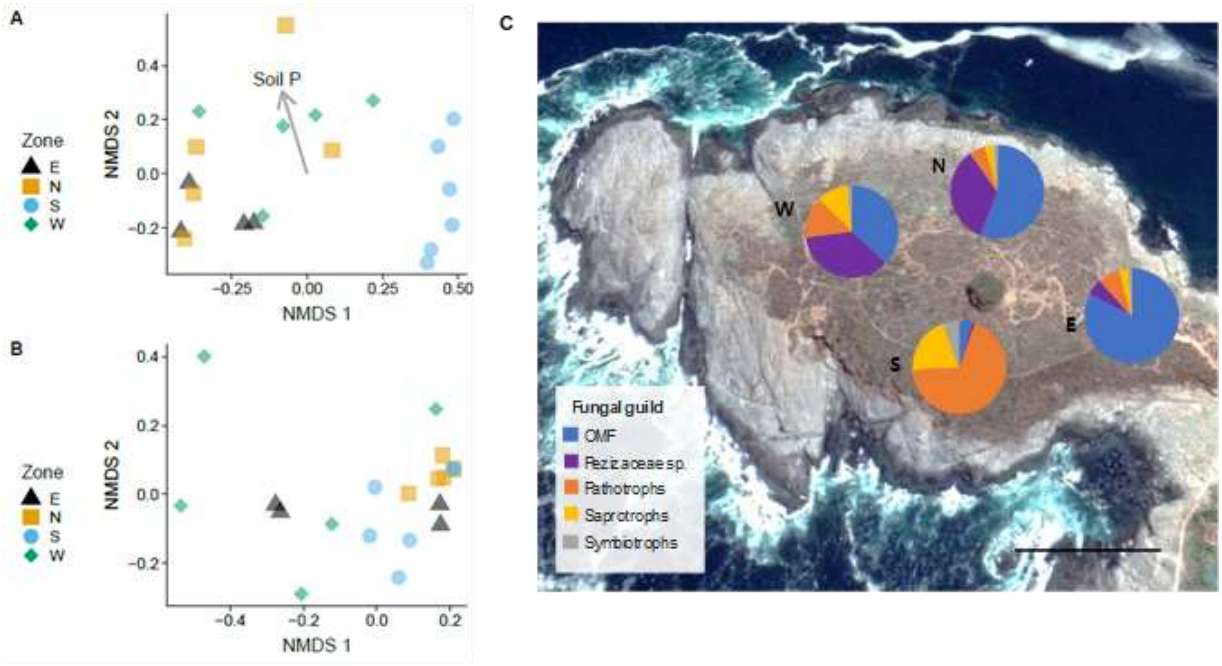

A
Soil P
Zone
E
N
S
W
NMDS 2
NMDS 1
B
C
N
W
E
S
Fungal guild
OMF
Rezizaceae sp.
Pathotrophs
Saprotrophs
Symbiotrophs

Figure 4.

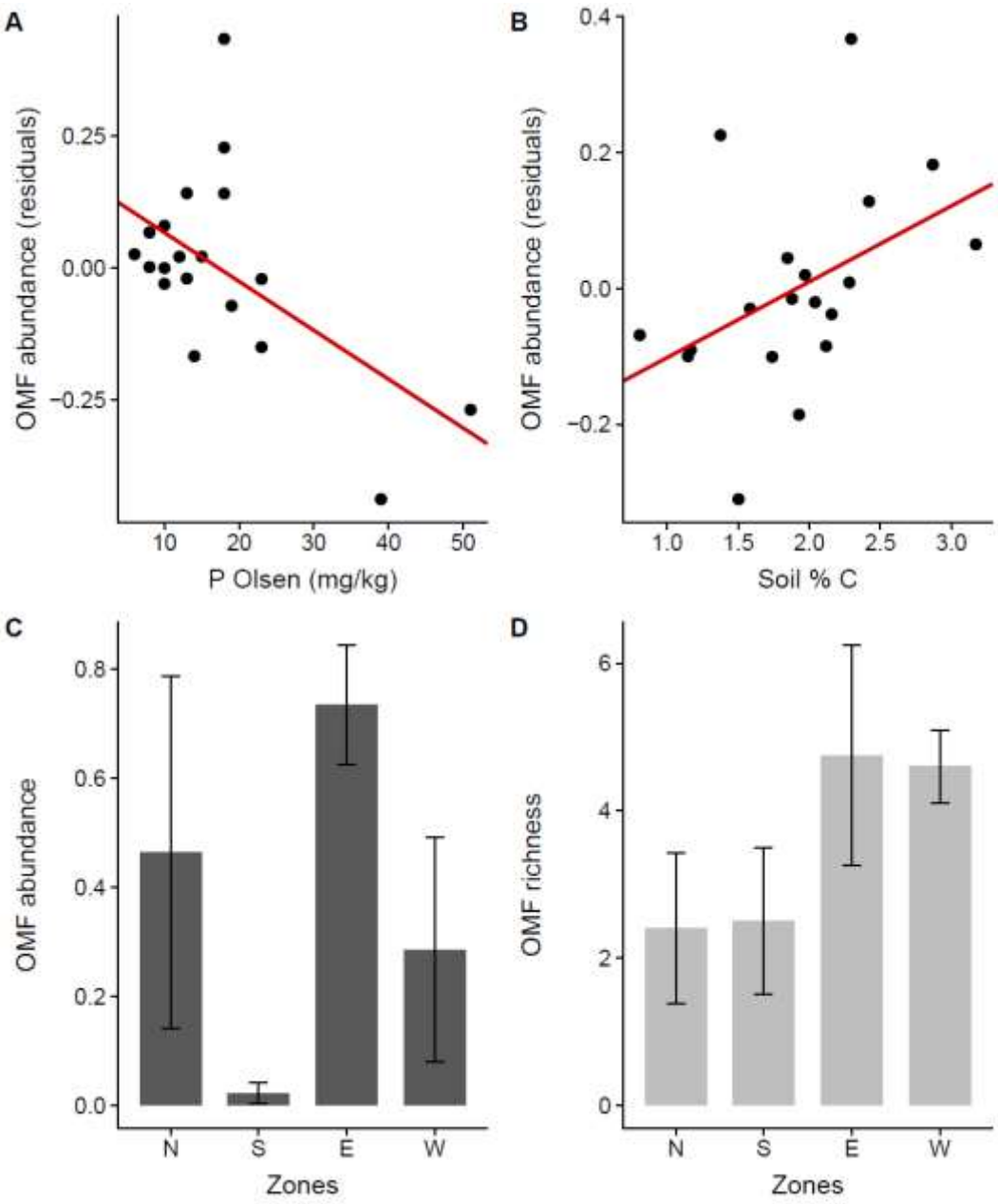

A
OMF abundance (residuals)
0.25
0.00
−0.25
10
20
30
40
50
P Olsen (mg/kg)
B
OMF abundance (residuals)
0.4
0.2
0.0
−0.2
1.0
1.5
2.0
2.5
3.0
Soil % C
C
OMF abundance
0.8
0.6
0.4
0.2
0.0
N
S
E
W
Zones
D
OMF richness
6
4
2
0
N
S
E
W
Zones

Figure 5.

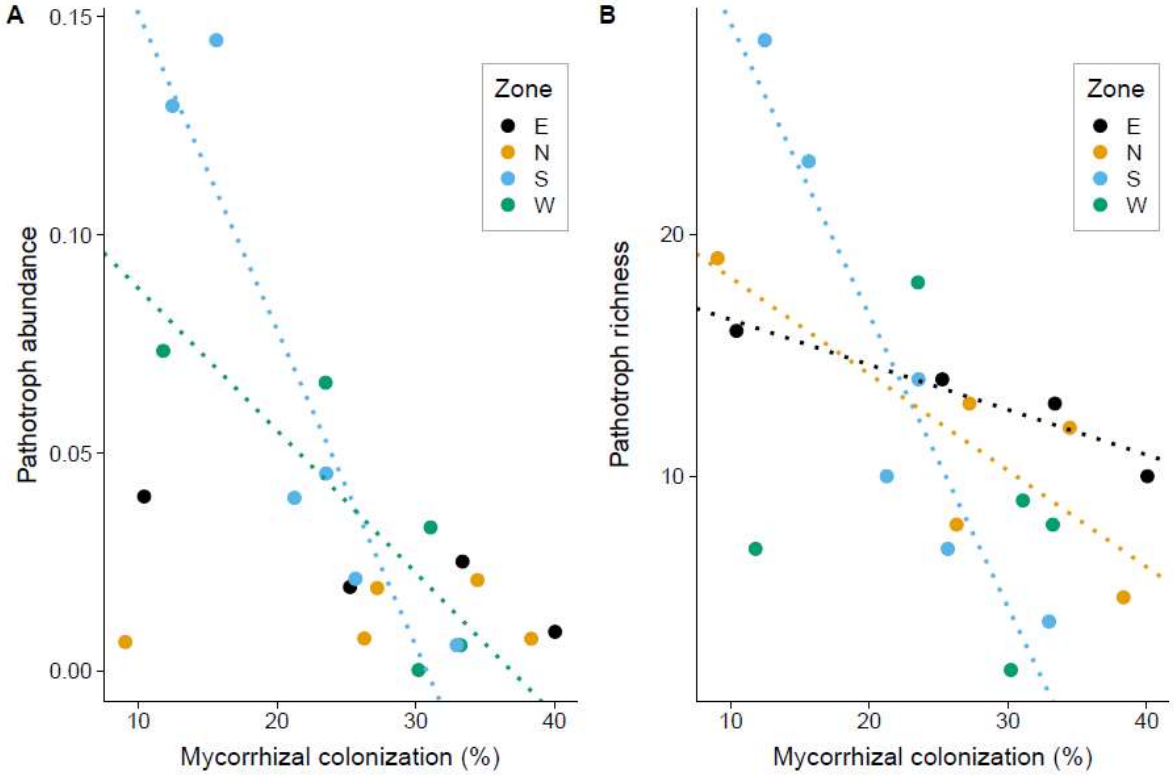
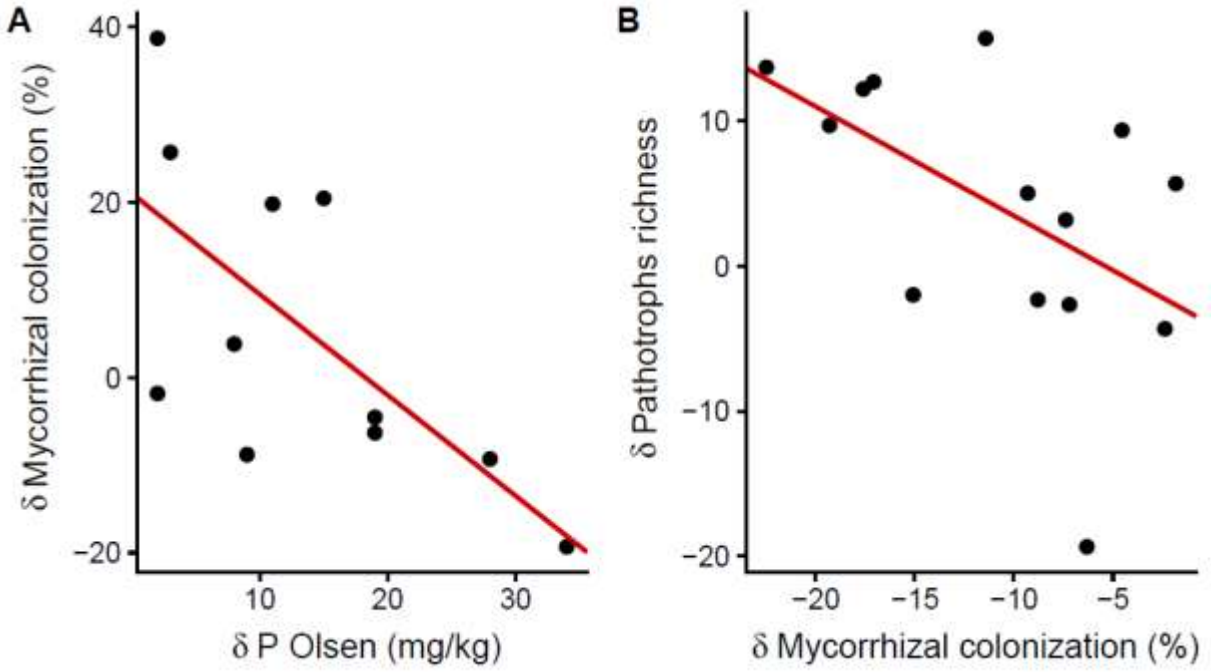

A
B
Zone
E
N
S
W
Pathotroph abundance
Pathotroph richness
Mycorrhizal colonization (%)
Mycorrhizal colonization (%)
0.15
0.10
0.05
0.00
20
10
10
20
30
40

Figure 6.


A
40
20
0
−20
δ Mycorrhizal colonization (%)
10
20
30
δ P Olsen (mg/kg)
B
10
0
−10
−20
δ Pathotrophs richness
−20
−15
−10
−5
δ Mycorrhizal colonization (%)

Capítulo III: Nutrients and fungal identity affect the outcome of symbiotic germination in *Bipinnula fimbriata* (Orchidaceae).

Title: Nutrients and fungal identity affect the outcome of symbiotic germination in *Bipinnula fimbriata* (Orchidaceae).

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ABSTRACT

Orchids produce tiny seeds without reserves, which depend entirely on orchid mycorrhizal fungi (OMF) for germination. This process, called symbiotic germination, can lead to different outcomes depending on abiotic factors such as nutrient availability. The underlying mechanism and whether this effect varies across OMF remains unknown. In this study we investigated the symbiotic germination of the orchid *Bipinnula fimbriata* with seven fungal OTUs from the families Tulasnellaceae and Ceratobasidiaceae, and evaluated the effect of fungal identity and nutrient addition on symbiotic germination and fungal growth rates. We found that fungal identity and nutrient availability are determinant for the outcome of symbiotic germination. Nevertheless, the effect of nutrient addition varied depending on the identity of the fungal partner involved, related to their growth response to nutrient addition. We discuss the possible mechanisms of these results and their implications for orchid ecology, and highlight the role of nutrients in orchid mycorrhizal associations.

Keywords: *Bipinnula*, *fungal identity*, *interaction outcomes*, *nutrient addition*, *Orchidaceae*, *orchid mycorrhiza*, *symbiotic germination*.

INTRODUCTION

One main question in the study of mutualisms is how the context influences the outcomes of mutualistic interactions (Johnson et al., 1997; Hoeksema and Bruna, 2015). Mutualisms are defined as interactions between species in which partners experience a net benefit (Bronstein, 2015). However, the costs and benefits that determine net effects can vary over time and space, also causing outcomes of interactions to vary along a continuum from mutualism to parasitism (Bronstein, 1994). This plasticity is frequently driven by the abiotic and biotic context in which the interaction occurs, such as the abundance of key nutrients and the identity of species present in the community (Hoeksema and Bruna, 2015).

Orchid mycorrhizas are an excellent model to study the variation of interaction outcomes (Bronstein et al., 2013). Orchidaceae, the largest family of Angiosperms (Chase et al., 2015), commonly form mycorrhizal associations with fungi belonging to three families (Tulasnellaceae, Sebacinaceae and Ceratobasidiaceae) of the Basidiomycetes (Dearnaley et al., 2012). Orchids produce numerous, tiny seeds lacking reserves (Arditti and Ghani, 2000; Barthlott et al., 2014), which rely entirely on orchid mycorrhizal fungi (OMF) for germination. In this process- known as symbiotic germination -the fungus colonizes the seed and they form a non-photosynthetic spherical body called the protocorm, which depends completely on the fungus for its nutrition (Rasmussen, 2002; Kuga et al., 2014). Seedlings then develop leaves, become autotrophic (in photosynthetic orchids) and nutrient exchange can occur between the mycorrhizal fungus and the orchid plant (Cameron, Leake and Read, 2006; Perotto et al., 2014). Nevertheless, the presence of OMF does not guarantee orchid germination by itself. The encounter of the seed with the mycorrhizal fungus can lead to the formation of

mycorrhizal protocorms (mutualistic outcome) or to the destruction of the seedlings (parasitic outcome) (Beyrle et al., 1995; Smith and Read, 2008), in a complex process influenced by OMF identity, biotic and abiotic factors (Rasmussen et al., 2015).

While the effect of OMF identity on orchid symbiotic germination has been widely demonstrated- especially in the case of specialist orchid, which only germinate with a restricted group of mycorrhizal fungi (Otero et al., 2004; Bonnardeaux et al., 2007; Swarts et al., 2010)- the effect of biotic and abiotic factors remains poorly understood. A few studies suggest that edaphic conditions may play an important role (Dijk et al., 1997). Using seed packets, Batty et al., (2001) found that soil potassium and presence of leaf litter was positively related to orchid seed germination, while Diez (2007) found that seed germination was influenced by soil moisture, organic matter and pH. Although seed baiting techniques do not distinguish whether soil factors have a direct effect on germination or an indirect effect mediated by affecting the abundance of OMF (McCormick et al., 2012), these results suggest a potential role of soil conditions in determining the result of symbiotic germination. In a more controlled experiment under *in vitro* conditions, Beyrle et al., (1991) observed that at low nitrogen (N) concentration the fungus *Rhizoctonia* sp. colonized seeds of the orchid *Dactylorrhiza incarnata* forming normal protocorms, but at high N concentration the fungus spread through orchid seeds, acting as a parasite and leading to protocorm destruction. The same negative effect of N addition was observed- but only at high levels of carbon supply - in the germination of the orchid *Orchis moro* with a *Rhizoctonia* species (Beyrle et al., 1995). These studies demonstrated the negative effect of nutrient addition on the outcome of orchid symbiotic germination, however the underlying mechanism and whether this effect varies across OMF species remains unknown.

There are two lines of evidence that suggest that the effect of nutrient addition on the outcome of orchid symbiotic germination should vary depending on fungal identity. First, because the diversity and composition of OMF associated with orchid species varies as a function of climatic and edaphic conditions (McCormick et al., 2006; Bunch et al., 2013, Mujica et al., 2016; Reiter et al., 2018). For example, Bunch et al. (2013) showed that the composition of mycorrhizal fungi varies among populations of the orchid *Cypripedium acaule* in association with soil pH, organic matter percentage, C and N. Similarly, Mujica et al. (2016) found a significant relationship between soil phosphorus (P) and nitrogen (N) and the composition and diversity of mycorrhizal fungi associated with two species of *Bipinnula.* These observations may be explained by different capabilities of OMF taxa to support orchids as a function of edaphic conditions (Mc Cormick et al., 2012; McCormick et al., 2018) and could be related to different germination abilities under different habitats. The second line of evidence is that OMF have different nutritional preferences among genera (Hadley and Ong, 1978; Nurfadilah et al., 2013; Fochi et al., 2017) and even among species of the same genus (Smith, 1967; Nurfadilah et al., 2013), which could cause different responses to nutrient addition in symbiotic germination. Considering this evidence, it is expected that the effect of nutrient addition on the outcome of orchid symbiotic germination will vary depending on OMF identity, and this variation will be related to different nutritional preferences of OMF species, but these hypotheses have not been tested yet.

In this study we addressed the effect of OMF identity, nutrient addition and their interaction on the outcome of orchid symbiotic germination. We expect nutrients to affect germination negatively, as was previously demonstrated (Beyrle et al., 1995), but also variation in the response to nutrients among different OMF species. Also, we expect that the variation in the

response to nutrients in symbiotic germination will be related to the direct effect of nutrients on fungal growth. To test this hypothesis, we evaluated (1) the effect of nutrient addition on symbiotic germination of *Bipinnula fimbriata*, a terrestrial and photosynthetic orchid endemic to the southern South America, with four OTUs of Ceratobasidiaceae and three OTUs of Tulasnellaceae isolated from adult plants of the same species, and (2) the effect of nutrient addition on the growth rates of these mycorrhizal fungi.

MATERIALS AND METHODS

Orchid species and seed collection

Bipinnula Comm. ex Juss. (subtribe Chloraeinae, subtribe Chloraeinae, Orchidoideae) is a genus of terrestrial, photosynthetic orchids endemic to southern South America. It comprises a separate group of five species endemic to Chile (Novoa et al., 2006; Cisternas et al., 2012). *Bipinnula fimbriata* (Poepp.) Johnst. is the most frequent of these five species; it is distributed in lowland (<500 m) coastal areas from 29 to 35°S (Novoa et al., 2006), preferably on sandy stabilized soils, in open sites exposed to sunlight and marine breezes (Elortegui and Novoa, 2009). Mature seeds were collected from three large populations of *B. fimbriata* located in Concón (32º33’ S), Zapallar (32º56’ S), and Los Vilos (31º58’ S). Seed capsules from these populations were mixed and dried at room temperature; seeds were stored in a glass vessel at 4 °C until sowing.

Mycorrhizal fungi

We sampled seven populations of *Bipinnula fimbriata* including its entire range of distribution. In each population we collected three roots from each of 10 individuals, which were kept cold until processed in the laboratory. Fungal associates were isolated from roots as

described in Steinfort et al., (2010). DNA from each fungal isolate was extracted, purified and sequenced as described in Mujica et al., (2016), and OTUs were defined by grouping sequences with more than 97% similarity. Among the isolated OTUs that corresponded to OMF, which belonged to the families Tulasnellaceae and Ceratobasidiaceae (Mujica et al., 2016), we chose the most frequent OTUs in the orchid populations, selecting four OTUs of the family Ceratobasidiaceae (named C1 to C4, Genbank accession nos. KP306714 KP306692, KP306722 and KP306721, respectively) and three OTUs of the family Tulasnellaceae (named T1 to T3, Genbank accession nos. KP306571, KP306672 and KP306574, respectively) to be used in the experiments (Fig.1).

Experimental Design

To evaluate the effect of fungal identity and nutrient treatment on symbiotic germination, seeds were sown with each fungal OTU under four different nutrient treatments based on OMA (oatmeal agar). OMA is a solid medium frequently used for orchid symbiotic germination (Janes, 2009) and has been successfully utilized in symbiotic germination of *Bipinnula fimbriata* (Steinfort et al., 2010; Herrera et al., 2016). The control treatment contained 3 g/L oat meal, 0.1 gr/L yeast extract and 7 g/l agar. The same components with addition of nitrogen (N) and phosphorus (P) in the forms NH_4NO_3 and KH_2PO_4 were used for the elaboration of enriched nutrient media (N, P and N+P) (Table 1). The concentrations used for N and P were based on Nurfadilah et al. (2013), who investigated the ability of orchid mycorrhizal fungi (OMF) to utilize a variety of nutrient sources. In each treatment 0.16mg/L streptomycin and 0.16mg/L penicillin were added, and pH was adjusted to 5.0–5.5 before

autoclaving, to reflect the soil pH of orchid populations from which mycorrhizal fungi were isolated.

Before sowing, seeds were disinfected by placing them in 2 mL Eppendorf tubes with 1.5 mL 1% sodium hypochlorite solution and five drops of Tween 20. The tubes were shaken for 3 min, then the solution was removed with a 5 mL sterile syringe. The seeds were washed three times with autoclaved distilled water, removing the water with a sterilized syringe each time. The sterilized seeds were resuspended in 1.5 mL of sterile distilled water and shaken to obtain a homogeneous suspension. The seeds were distributed in Petri dishes and then a mycelium plug was transferred to the center of each dish. Each nutritional treatment with each fungal OTU, including a control without mycorrhizal fungi, was replicated ten times. Petri dishes were stored in a dark room at 18 °C. Germination was evaluated 30 days after sowing, recording the percentage of seeds in each stage of germination as: 0, Intact seeds; 1, Coat rupture; 2, Formation of rhizoids (Mitchel, 1988).

The same nutrient treatments (Control, N, P and N+P) were used to evaluate the effect of nutrient concentration on fungal growth. A 0.25 cm^2 plug of mycelium from fungal culture was transferred to the center of Petri dishes containing the media treatments, replicated ten times. Plates were placed in a dark room at 18 °C. Fungal growth was measured daily by marking and photographing the mycelium extension, calculating the area of the mycelium with the software ImageJ (Rueden et al., 2017). The plates were measured until the mycelium covered the entire Petri dish or after 40 days of growth. Growth rate was estimated as the mean difference between daily area measurements during the exponential phase of the growth curves.

Statistical analyses

A one-way ANOVA was performed to test the effect of fungal identity on percentage of seeds in stage 1 (coat rupture) and percentage of seeds in stage 2 (formation of rhizoids) in the control treatment (without nutrient addition). A two-way ANOVA was performed to test the effect of fungal identity and nutrient treatment on percentage germination, with fungal identity and treatments as independent factors, including the interaction between them, and percentage germination in stage 2 as the response variable. Similarly, a two-way ANOVA was utilized to test the effect of fungal identity and treatment on growth rate. A Tukey test for *a posteriori* analyses was conducted after each ANOVA. Analyses were performed in R software (R Core Team; 2018)

RESULTS

After 30 days of culture seeds presented the first stage (rupture of coat) or the second stage (formation of rhizoids) of germination. In the control treatment the first stage of germination was observed in the presence of the seven fungal OTUs and in the plates without mycorrhizal fungi, but there were significant differences among them ($F = 10.1$, $P = 3e^{-08}$; Fig. 2A). The second stage was observed only in the presence of mycorrhizal fungi, whereas in plates without fungi there was no formation of rhizoids. There were significant differences in rhizoid formation among the different mycorrhizal OTUs ($F = 18.8$, $P = 5.9e^{-13}$), C1 presented the highest percentage of formation of rhizoids, while T1, C4 and T2 had the lowest percentages (Fig.2B).

The factors fungal identity and treatment had a significant effect on percentage of rhizoid formation (Table 2). The interaction between fungal identity and treatments was also

significant, showing that the effect of nutrients on symbiotic germination varied among fungal OTUs. In the control treatment all mycorrhizal fungi promoted the development of rhizoids (Fig.2B); under nutrient addition treatments (P, N+P and N), this only occurred in plates with OTUs C1 and C2 (Fig.3). OTU C1 presented no differences in rhizoid formation among the four treatments, while C2 showed significantly higher germination in the control treatment, followed by N, without significant differences between the N+P and P treatments. The formation of rhizoids was near zero under nutrient addition with OTUs C3, C4, T1, T2 and T3, with no significant differences among nutrient treatments (N, P, N+P). In these treatments these fungal OTUs grew through the seeds and germination did not continue further.

There was a significant effect of fungal identity, nutrient treatment and the interaction between them on fungal growth rates (Table 2). Nutrient treatments significantly affected growth of all fungal OTUs except for C1 and C2, which showed no significant differences among treatments (Fig.4). OTUs C3, C4, T1 and T3 presented the same pattern of growth under treatments, showing a negative effect of N addition on growth rate, where the highest growth rate was observed in the control (N addition= 0 gr/l), followed by a similar growth rate in N+P and P (N addition= 0.3 gr/l), and the lowest growth rate under N treatment (N addition= 0.6 gr/l), (Fig.3). Lastly, T2 presented a different pattern, with the lowest growth rate in the control and no significant differences among N, P and N+P (Fig 4).

DISCUSSION

In this study we found that fungal identity and nutrient concentration are determinant for the outcome of symbiotic germination of *Bipinnula fimbriata*, however, the effect of nutrient addition on symbiotic germination depended on the identity of the fungal partner involved.

Outcomes of symbiotic germination

In orchid symbiotic germination, hyphal penetration may occur through rhizoids or through the seed suspensor, depending on the orchid species (Rasmussen, 1995). In the former case seeds can produce rhizoids before the fungal invasion occurs, then seedlings can survive while the nutrient stores last, or until a fungus penetrates through rhizoids and further assists development. In the latter case, seeds will only develop rhizoids after invasion through the suspensor (Rasmussen and Rasmussen, 2014). This study and a previous report found the absence of rhizoid formation in plates with no mycorrhizal fungi in *Bipinnula fimbriata* (Steinfort et al., 2010) suggesting that hyphal penetration occurs through the seed suspensor in this species. Swelling of the embryo and rupture of the seed coat were observed in all plates, regardless of treatment media, fungal presence or identity, probably related to seed water uptake (Smith, 1967). After this initial stage, the interaction between *B. fimbriata* seeds and mycorrhizal fungi had two possible outcomes: the mutualistic outcome, where the seedlings are penetrated by hyphae and form rhizoids, or the parasitic outcome (according to Beyrle et al., 1995), where fungus grows through the seed without rhizoid formation and there is no further development of the seeds.

Fungal identity

Bipinnula fimbriata was able to germinate (form rhizoids) with the seven fungal OTUs tested in this study, suggesting the absence of mycorrhizal specificity in this orchid species. This agrees with other studies on *B. fimbriata* germination that showed lack of specificity (Steinfort et al., 2010; Herrera et al., 2016). However, fungal identity had a significant effect on germination; C1 (*Ceratobasidium* sp.) presented the highest percentages of germination and

T1 the lowest (*Tulasnella* sp.). This differs from the results obtained by Herrera et al. (2016) who observed more germination of *B. fimbriata* seeds with *Tulasnella* species. This difference could be due to local adaptation of seeds to different mycorrhizal fungi, as the seeds used in this study were obtained from northern populations of *B. fimbriata* and in Herrera et al. (2016) they were obtained from south-central populations. Further studies assessing the effect of local adaptation would contribute to understand these differences. Besides this difference, both results suggest that fungal identity affects the result of the symbiosis between orchid and mycorrhizal fungi.

Nutrient addition

Similar to the findings of Beyrle et al. (1991), the addition of nutrients in *Bipinnula fimbriata* turns the interaction between orchid seeds and mycorrhizal fungi from mutualistic to parasitic in most fungal species. Five out of the seven fungal species showed a negative effect of nutrient addition on rhizoid formation. Remarkably, there were no differences among the three enriched treatments (N+P, N and P) on symbiotic germination with these five fungi (Fig. 3). This suggests that the concentration of nutrients in the N+P treatment was enough to inhibit the germination of *B. fimbriata* and there was no effect of the additional increase of N or P. The effect of nutrients on symbiotic germination could be related to a direct effect of nutrients on seeds. Studies on asymbiotic germination might offer some clues; Ponert et al., (2013) demonstrated that nitrate had a negative effect on asymbiotic germination of *Pseudorchis albida* even at extremely low concentration. These results suggest that the observed effect of nutrient addition on symbiotic germination could be mediated by a direct effect of nitrate on seeds. The three enriched treatments used nitrate at inhibitory levels (Table 1). However, we

observed that the effect of nutrients on symbiotic germination was not the same for all mycorrhizal fungi, but rather varied among fungal species. In particular, there was no effect of nutrient addition on symbiotic germination with OTU C1 (*Ceratobasidium* sp.) and a weaker effect on C2. A similar pattern was observed in the orchids *Gymnadenia conopsea* and *Dactylorhiza majis* (Tomas Figura, pers. comm.), where the effect of nitrate addition on symbiotic germination varied depending on fungal identity. Similar to our observations, a negative effect of nitrate was observed for *Tulasnella* and *Sebacina*, but not for *Ceratobasidium*. It is possible that there is an inhibitory effect of nitrate on germination, but it is eliminated by some fungal species such as *Ceratobasidium* species (Tomas Figura, pers. comm.), which would be supported by the results of this study.

Fungal nutrition

Alternatively, the effect of nutrients on symbiotic germination outcome might be mediated by a direct effect on fungal nutrition (Beyrle et al., 1995). Orchid mycorrhizal fungi have different nutritional preferences, for example, *Tulasnella* species can use ammonium but not nitrate as inorganic N form, whereas *Ceratobasidium* can use both ammonium and nitrate (Nurfadilah et al., 2013), which may be explained by the lack of genes involved in nitrate uptake and reduction in the *T. calospora* genome (Fochi et al., 2017). Our results also showed different responses to nutrients among fungal species. The growth of the OTUs C1 and C2 (*Ceratobasidium* spp.) were not affected by nutrient treatments, whereas the growth rates of C3, C4 (*Ceratobasidium* spp.), T1 and T3 (*Tulasnella* spp.) were affected negatively by nitrogen addition (Fig.3). The measure of mycelium extension does not give direct information about fungal biomass, but it is a direct measurement of the rate of hyphal elongation, where

higher rates indicate faster expansion of the mycelium through the plate. Filamentous fungi ramify into evenly dispersed mycelia and exploit nutrients in the substratum maximally (Brand and Gow, 2009), thus higher rates of mycelium extension suggest searching for nutrients in the culture medium. Thus the higher growth rates in the control treatment with C3, C4, T1 and T3 might suggest nutrient limitation in these fungal species, which decreases with addition of nitrogen (Fig 3). The control treatment was the only treatment where these species presented formation of rhizoids, suggesting that these fungi are capable of germinating the seeds of *B. fimbriata* only under nutrient limitation conditions.

The variation in growth response to nutrients among fungal species is correlated with the variation in the effect of nutrients on symbiotic germination. C1 and C2 (*Ceratobasidium* spp.) were the only fungi with no effect of nutrients on fungal growth and were also the only OTUs that could germinate seeds with nutrient addition. The growth rate in the other five OTUs was influenced by nutrient addition and the symbiotic germination was inhibited by nutrient addition, presenting zero protocorm formation under enriched treatments. Although the mechanism remains unclear, these results suggest that the effect of nutrients on symbiotic germination outcome might be partly explained by a direct effect of nutrients on fungal nutrition. In addition, C1 and C2, the OTUs that had a similar response to nutrients in growth and symbiotic germination, are also phylogenetically closer than the other OTUs (Fig.1) suggesting that these responses might be phylogenetically conserved.

Conclusions

In this study we demonstrated that nutrients influence symbiotic germination and that their effect varies depending on mycorrhizal fungal identity. It is still unknown if this effect is

mediated by an inhibitory effect of nutrients on seeds that is precluded in some fungal species (Tomas Figura, pers. comm.) or if it is mediated by fungal nutrition that depends on fungal identity. The variation in responses to nutrients of fungal species in symbiotic germination could have important ecological implications for orchid mycorrhizal associations. For example, a mycorrhizal fungus that is capable of germinating orchid seeds in a broader range of nutrient availability should be a suitable partner in a wider range of habitats. This was observed for OTU C1, which can germinate seeds equally under low and high nutrient concentrations (Fig.3) and is present in a wider range of habitats associated with *Bipinnula* (Mujica et al., 2016). Thus, further research on factors affecting orchid symbiotic germination outcomes is needed for a better understanding of orchid ecology and successful conservation strategies. Finally, this study supports the idea that nutrients play an important role in orchid mycorrhizae (Dijk et al., 1997), and provides new questions for future research in exploring the mechanisms that underlie orchid symbiotic germination.

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FIGURE LEGENDS

Figure 1. Phylogenetic relationships among the OTUs of (A) Ceratobasidiaceae and (B) Tulasnellaceae used in this study. Trees were constructed based on internal transcribed spacer (ITS) fungal sequences obtained from *Bipinnula fimbriata* and *B. plumosa* roots, and Genbank accessions (Details in Mujica et al., 2016). Both trees are the Bayesian majority consensus trees, with values on each branch indicating parsimony bootstrap values/maximum likelihood bootstrap values/Bayesian posterior probabilities.

Figure 2. Percentage of *Bipinnula fimbriata* seeds in the two first stages of orchid symbiotic germination (A) Seed coat rupture and (B) Formation of rhizoids of *Bipinnula fimbriata* seeds, with seven OTUs of orchid mycorrhizal fungi and a control without mycorrhizal fungi in oatmeal agar (OMA) media. C1 to C4 are Ceratobasidiaceae OTUs and T1 to T3 are Tulasnellaceae OTUs. One-way ANOVAs were performed for each stage of germination and significant differences of the *a posteriori* Tukey test are shown in lowercase letters.

Figure 3. Percentage of seeds of *Bipinnula fimbriata* with formation of rhizoids, under four nutritional treatments with seven mycorrhizal fungal OTUs and a control without mycorrhizal fungi. Each panel corresponds to a mycorrhizal OTU; C1 to C4 are Ceratobasidiaceae and T1 to T3 are Tulasnellaceae. Treatments are control (C), enriched in nitrogen and phosphorus (N+P), enriched in phosphorus (P), and enriched in nitrogen (N). A two-way ANOVA was performed and significant differences of the *a posteriori* Tukey test are shown in lowercase letters. ns= not significant.

Figure 4. Growth rate of the seven mycorrhizal OTUs isolated from *Bipinnula fimbriata* roots under four nutritional treatments. Treatments are control (C), enriched in nitrogen and

phosphorus (N+P), enriched in phosphorus (P), and enriched in nitrogen (N). Each panel corresponds to a mycorrhizal OTU; C1 to C4 are Ceratobasidiaceae and T1 to T3 are Tulasnellaceae. A two-ways ANOVA was performed and significant differences of the *a posteriori* Tukey test are shown in lowercase letters. ns= not significant

Table 1. Components of the nutrient treatments used in the germination and fungal growth experiments

Component	Treatment			
(gr/lt)	Control	NP	P	N
Oatmeal	3	3	3	3
Yeast extract	0.1	0.1	0.1	0.1
Agar	7	7	7	7
NH_4NO_3	0	0.3 (3.7 mM N)	0.3 (3.7 mM N)	0.6 (7.5 mM N)
KH_2PO_4	0	0.3 (2.3 mM P)	0.6 (4.5 mM P)	0.3 (2.3 mM P)

Table 2. Effect of fungal identity, Treatments and the interaction between them on the symbiotic germination (measured as rhizoid formation) of *Bipinnula fimbriata* and on the growth of the seven mycorrhizal fungi species.

Source of Variation	F value	P value
ANOVA 1: Effect on germination (rhizoids formation)		
Fungal Identity	239	<2e-16
Treatment	259	<2e-16
Fungal Identity*Treatment	12	<2e-16
ANOVA 2: Effect on fungal growth		
Fungal Identity	294	<2e-16
Treatment	127	<2e-16
Fungal Identity*Treatment	34	<2e-16

Figure 1.

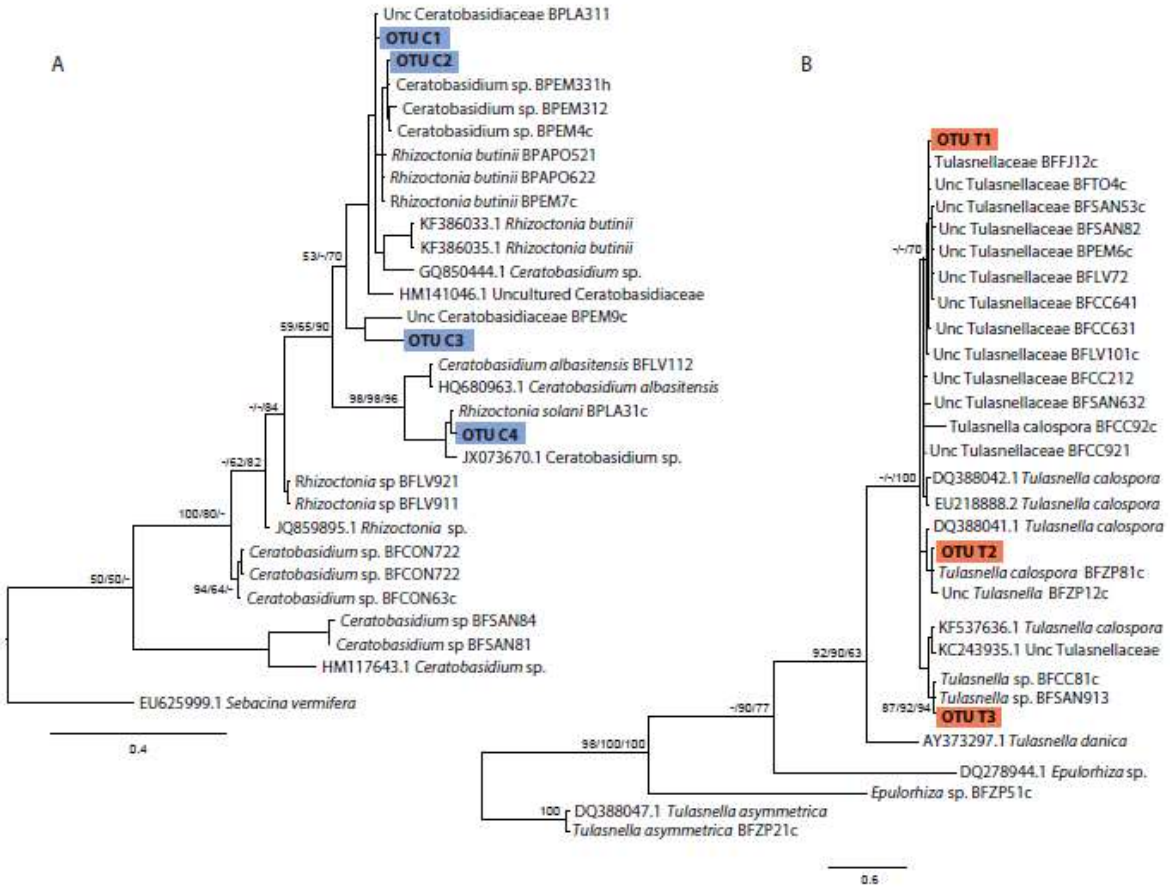

A
Unc Ceratobasidiaceae BPLA311
OTU C1
OTU C2
Ceratobasidium sp. BPEM331h
Ceratobasidium sp. BPEM312
Ceratobasidium sp. BPEM4c
Rhizoctonia butinii BPAPO521
Rhizoctonia butinii BPAPO622
Rhizoctonia butinii BPEM7c
KF386033.1 Rhizoctonia butinii
KF386035.1 Rhizoctonia butinii
GQ850444.1 Ceratobasidium sp.
HM141046.1 Uncultured Ceratobasidiaceae
Unc Ceratobasidiaceae BPEM9c
OTU C3
Ceratobasidium albasitensis BFLV112
HQ680963.1 Ceratobasidium albasitensis
Rhizoctonia solani BPLA31c
OTU C4
JX073670.1 Ceratobasidium sp.
Rhizoctonia sp BFLV921
Rhizoctonia sp BFLV911
JQ859895.1 Rhizoctonia sp.
Ceratobasidium sp. BFCON722
Ceratobasidium sp. BFCON722
Ceratobasidium sp. BFCON63c
Ceratobasidium sp BFSAN84
Ceratobasidium sp BFSAN81
HM117643.1 Ceratobasidium sp.
EU625999.1 Sebacina vermifera
0.4
B
OTU T1
Tulasnellaceae BFFJ12c
Unc Tulasnellaceae BFTO4c
Unc Tulasnellaceae BFSAN53c
Unc Tulasnellaceae BFSAN82
Unc Tulasnellaceae BPEM6c
Unc Tulasnellaceae BFLV72
Unc Tulasnellaceae BFCC641
Unc Tulasnellaceae BFCC631
Unc Tulasnellaceae BFLV101c
Unc Tulasnellaceae BFCC212
Unc Tulasnellaceae BFSAN632
Tulasnella calospora BFCC92c
Unc Tulasnellaceae BFCC921
DQ388042.1 Tulasnella calospora
EU218888.2 Tulasnella calospora
DQ388041.1 Tulasnella calospora
OTU T2
Tulasnella calospora BFZP81c
Unc Tulasnella BFZP12c
KF537636.1 Tulasnella calospora
KC243935.1 Unc Tulasnellaceae
Tulasnella sp. BFCC81c
Tulasnella sp. BFSAN913
OTU T3
AY373297.1 Tulasnella danica
DQ278944.1 Epulorhiza sp.
Epulorhiza sp. BFZP51c
DQ388047.1 Tulasnella asymmetrica
Tulasnella asymmetrica BFZP21c
0.6

Figure 2.

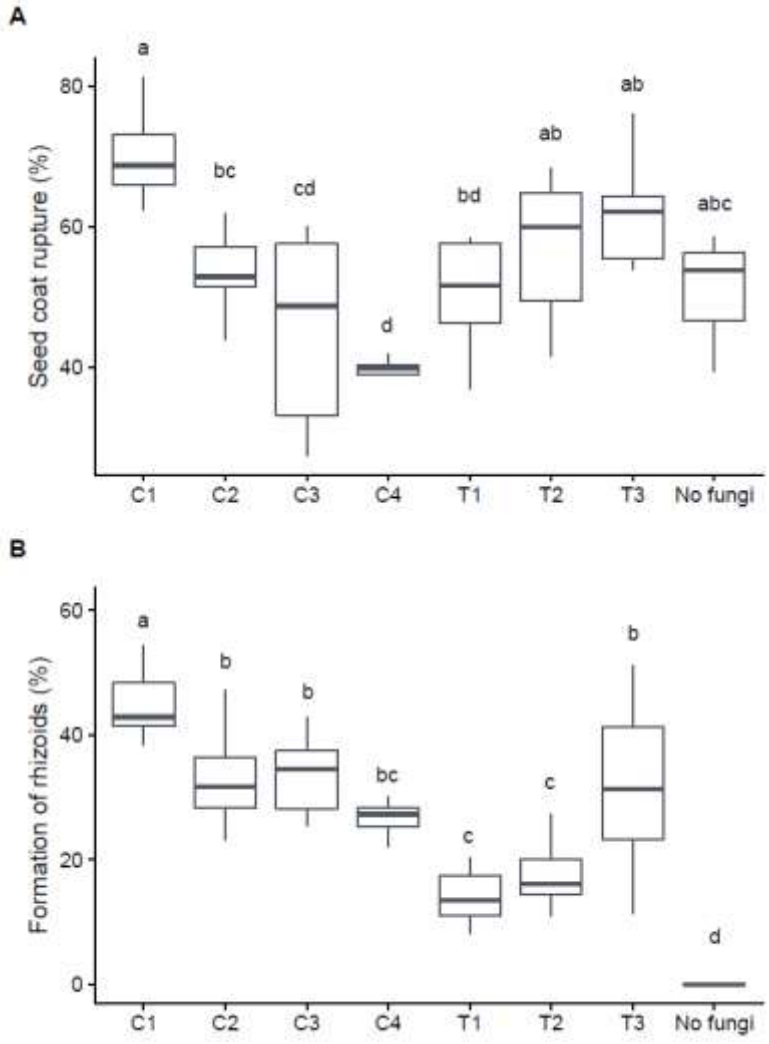

A
Seed coat rupture (%)
80
60
40
a
bc
cd
d
bd
ab
ab
abc
C1
C2
C3
C4
T1
T2
T3
No fungi
B
Formation of rhizoids (%)
60
40
20
0
a
b
b
bc
c
c
b
d
C1
C2
C3
C4
T1
T2
T3
No fungi

Figure 3.

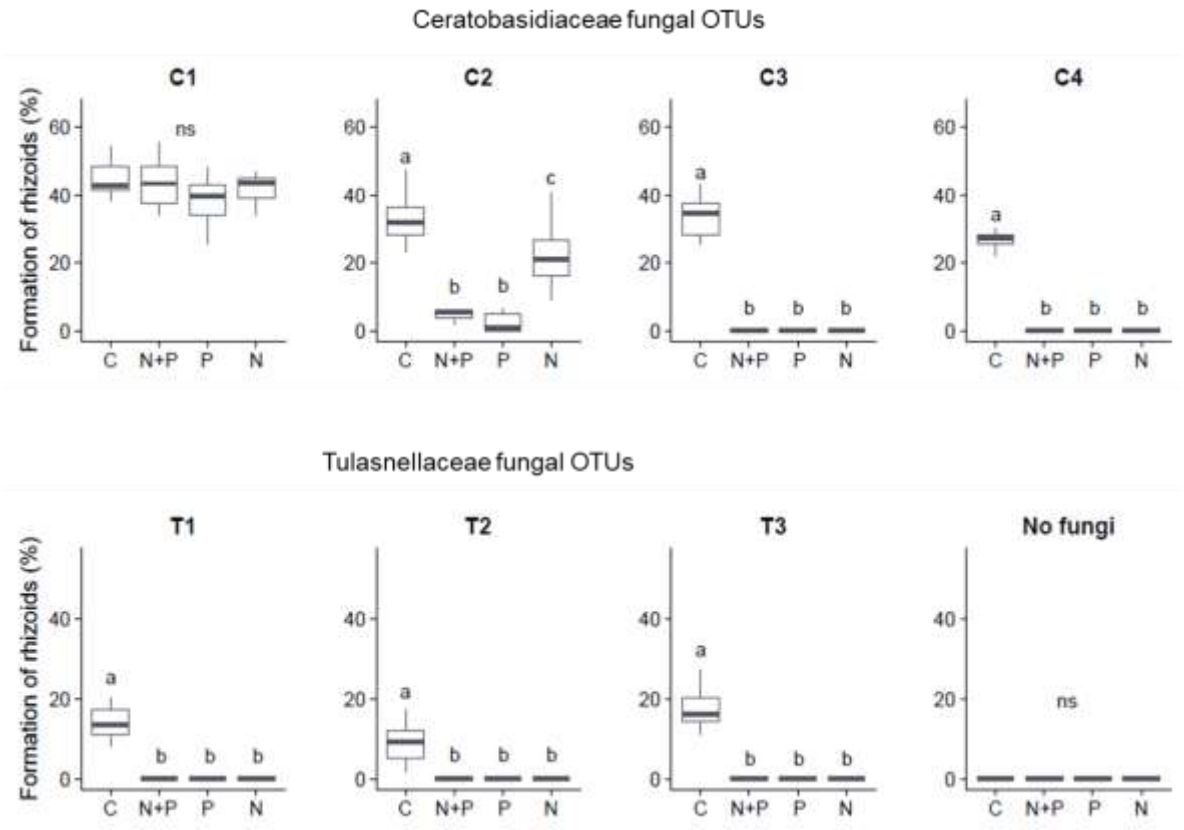
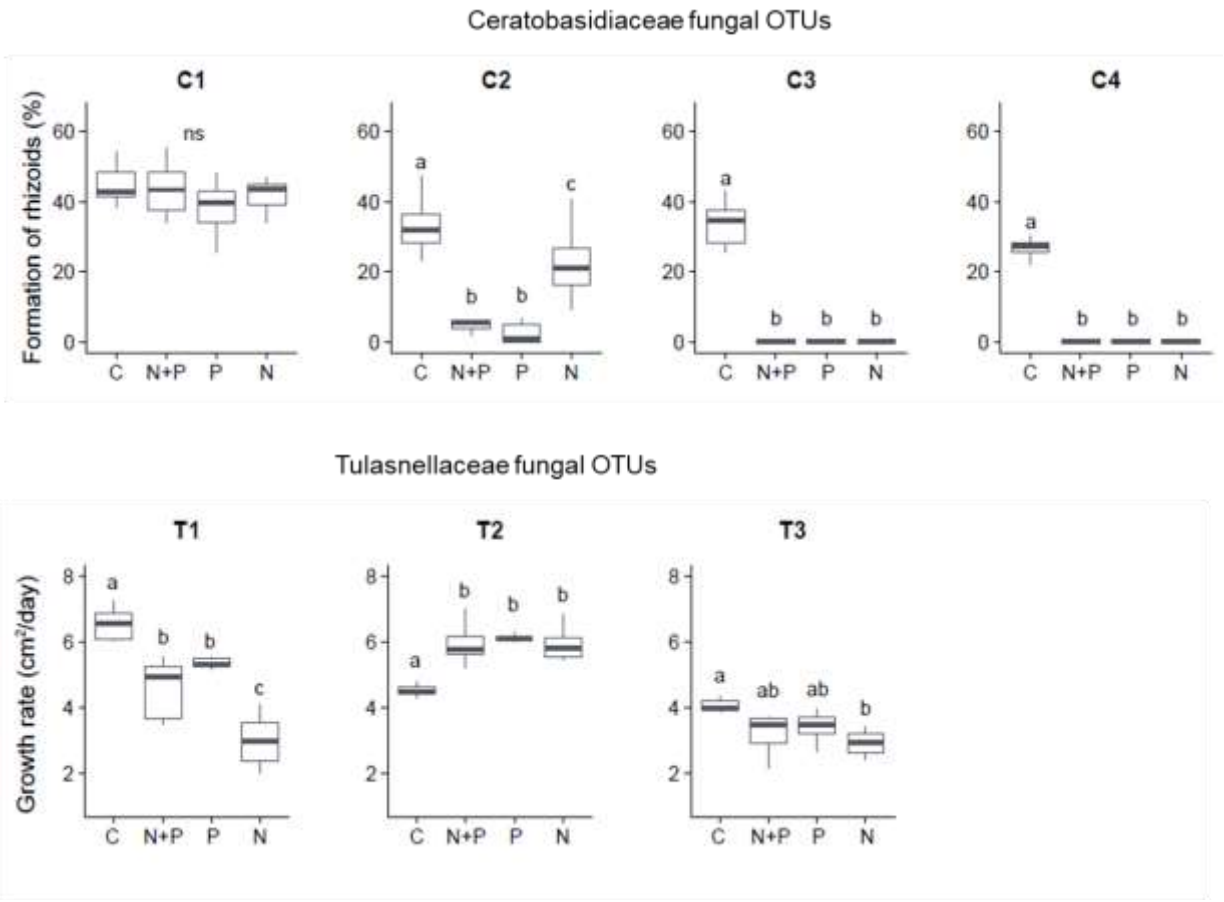

Ceratobasidiaceae fungal OTUs
C1
C2
C3
C4
Formation of rhizoids (%)
ns
a
b
c
C
N+P
P
N
Tulasnellaceae fungal OTUs
T1
T2
T3
No fungi

Figure 4.


Ceratobasidiaceae fungal OTUs
C1
C2
C3
C4
Formation of rhizoids (%)
ns
a
b
c
C
N+P
P
N
Tulasnellaceae fungal OTUs
T1
T2
T3
Growth rate (cm²/day)
ab

CONCLUSIONES GENERALES

En esta tesis se estudió la especialización en las asociaciones micorrícicas desde diferentes perspectivas y a distintos niveles de organización biológica. Desde el estudio de la evolución de las micorrizas utilizando de métodos de macroevolución, hasta el estudio de un tipo particular de micorrizas, las micorrizas de orquídeas, desde una aproximación comunitaria y experimental, hasta el análisis de los factores que influyen en el encuentro del hongo micorrícico con la semilla de la orquídea.

A una escala macroevolutiva, el estudio de las micorrizas puede ayudar a comprender la evolución de las plantas, ya que las asociaciones micorrícicas podrían afectar sus tasas de diversificación. Por primera vez se puso a prueba esta hipótesis y se demostró que aquellas familias de plantas con una mayor diversidad de tipos de micorriza tenían mayores tasas de diversificación. Estos resultados respaldan la hipótesis que sugiere que una mayor plasticidad en los tipos de micorrizas promueve la diversificación, que había sido planteada para otros mutualismos; y además reafirman la idea de que la especialización representaría una desventaja a escala evolutiva. Por lo tanto, muestran la importancia de comprender qué factores promueven la especialización a una escala ecológica a pesar de estas desventajas evolutivas.

Para evaluar estos factores ecológicos, a una escala comunitaria y utilizando secuenciación masiva, se evaluó la relación entre los nutrientes del suelo y la especialización micorrícica en la orquídea *Bipinnula fimbriata*. En primer lugar, este estudio permitió identificar una gran diversidad hongos asociados a las raíces de esta especie, incluyendo hongos micorrícicos, patógenos, saprófitos y endófitos, cuyas funciones en las raíces de las

orquídeas aún son desconocidas. En segundo lugar, se observó que el fósforo del suelo se relacionaba negativamente con la abundancia de hongos micorrícicos, lo que sugiere que ante bajas disponibilidades de fósforo, la asociación se intensifica. Consecuentemente, la adición de fósforo impactó negativamente a la colonización micorrícica. Sin embargo, no hubo efectos sobre la diversidad de hongos, lo que sugiere que, al menos a esta escala, la planta reacciona a la adición del fósforo modulando la intensidad de la interacción, más que el nivel de especialización o la composición de hongos micorrícicos. Por otro lado, se observó una relación negativa entre colonización micorrícica y la abundancia y riqueza de patógenos, que había sido descrita en otros tipos de micorrizas, pero por primera vez se observa en micorrizas de orquídeas. Asimismo, la disminución en la colonización micorrícica producida por el aumento del fósforo, produjo un aumento en la riqueza de hongos patógenos, confirmando un posible papel de la colonización de micorrizas en la defensa contra patógenos. Así, se demostró que en este sistema los nutrientes del suelo modulan la asociación entre orquídeas y hongos micorrícicos, y que esto tiene efectos sobre la comunidad de hongos no-micorrícicos.

Si la adición de nutrientes no tuvo un efecto en la especialización ni en la composición de hongos micorrícicos asociados a plantas adultas de *B. fimbriata*, la disponibilidad de nutrientes podría tener un papel importante en la etapa de la germinación, donde ocurre el encuentro inicial entre el hongo micorrícico y la orquídea. Efectivamente, se observó que la adición de nutrientes afecta negativamente la germinación simbiótica, pero que este efecto depende de la identidad del hongo involucrado. Además, se observó que la respuesta del hongo a la adición de nutrientes en la germinación simbiótica depende de sus requerimientos nutricionales. Estos resultados sugieren que la disponibilidad de nutrientes podría determinar

la composición de hongos con los que una orquídea interactúa desde las etapas iniciales, posiblemente influenciando la diversidad de hongos observados en las plantas adultas.

Los resultados obtenidos en cada aproximación abren nuevas interrogantes y proponen nuevos desafíos para el estudio de las micorrizas. Por ejemplo, la relación positiva entre la diversidad de tipos de micorrizas y las tasas de diversificación en las familias de plantas con semillas, abre nuevas preguntas como ¿se observa este patrón a menores niveles taxonómicos? Esto requiere la disponibilidad de filogenias de plantas más resueltas a nivel de especie. Además, si una mayor diversidad de tipos de micorrizas permite colonizar nuevos ambientes, ¿las familias que tienen mayor diversidad de tipos de micorrizas tienen también distribuciones geográficas más amplias? Por otra parte, se observó una correlación negativa entre la colonización por micorrizas y la abundancia y riqueza de patógenos. ¿Cuál es el mecanismo que explica esta correlación? Experimentos de cultivo *in vitro* podrían contribuir para entender la interacción que ocurre entre estos hongos dentro de la raíz. Por último, se observó que el efecto negativo de la adición de nutrientes sobre la germinación depende de la identidad del hongo micorrícico involucrado. En particular, hay especies de hongos micorrícicos que no son afectados por los nutrientes y otros que sólo germinan cuando hay baja disponibilidad de nutrientes. Este sistema de estudio es ideal para explorar los mecanismos subyacentes a la germinación simbiótica de las orquídeas ¿Qué ocurre con el hongo cuando hay baja disponibilidad de nutrientes, que sólo en esas condiciones logra germinar a las semillas? Estudios en expresión génica de los hongos y de las semillas podrían entregar pistas para entender este enigma.

En conclusión, utilizando diferentes aproximaciones, esta tesis contribuye con nuevos conocimientos para responder a las principales preguntas sobre la especialización. Se muestra que, en el caso de las micorrizas, la especialización puede tener consecuencias sobre la evolución de las plantas, afectando sus tasas de diversificación; y que la asociación entre plantas y hongos micorrícicos es influenciada por factores ecológicos como la disponibilidad de nutrientes en el suelo. Los resultados de esta tesis plantean la necesidad de estudios que combinen ambas aproximaciones, estudiando por ejemplo, las tasas de diversificación de linajes en ambientes donde se favorezca la especialización; y abren nuevas interrogantes para el estudio de las micorrizas. Finalmente, en esta tesis se reafirma el rol clave que ha tenido la asociación micorrícica para las plantas, desde su influencia en la diversificación de éstas, hasta su importancia para la germinación, crecimiento y sobrevivencia, en el caso particular de las orquídeas.